The Immunobiology of Scrapie Following Transmission via the Skin

Joanne Mohan

A thesis submitted in partial fulfilment of the requirements of the University of Edinburgh for the degree of Doctor of Philosophy

The programme of research was carried out at the Institute for Animal Health, Neuropathogenesis Unit, Edinburgh.

October 2004

Declaration

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed by myself, in collaboration with my supervisors Dr Neil Mabbott, Prof Moira Bruce and Prof John Hopkins, unless otherwise stated. No part of this work has been, or will be submitted for any other degree, or professional qualification.

Joanne Mohan

October 2004

Acknowledgements

I would like to thank my supervisors **Neil Mabbott, John Hopkins** and **Moira Bruce** for their support, mentoring and guidance over the past three years. I would especially like to thank Neil Mabbott whose patience, red pen and wit got me through this. I would also like to thank all members of staff at NPU for all their help and support.

I would like to express my gratitude to the following members of staff for their technical support during the course of the project;

Irene McConnell, Mary Brady, Simon Cumming, Lorraine Gray, Fraser Laing, Rebecca Greenan, Leeann Frame (NPU animal facility)- for assistance provided with animal husbandry.

Karen Brown- for carrying out the bone-marrow reconstitutions as detailed in Chapter 4

Wing-gee Liu and **Aileen Boyle**- for vacuolation scoring

Anne Suttie, Sandra Cooper, Gillian McGregor and **Sandra Mack**- for all their histology support.

I would like to thank the following individuals who supplied reagents, mouse models or advice.

Prof David Gray, (University of Edinburgh, Edinburgh, UK) for the provision of the CD40 Ligand knockout mouse model.

Prof Man-Sun Sy, (Case Western Reserve University, School of Medicine, Cleveland, OH, USA) for the provision of the PrP-specific antibodies 8H4 and 7A12.

Prof Akira Takashima, (Dept. Dermatology, University of Texas, Texas, USA) for provision of the XS106, XS52 and NS47 cell lines.

Dr. Jeffrey Browning (Biogen Inc., Cambridge, MA, USA) for provision of the LTβR-Ig reagent.

Prof Richard Groves (Imperial College, London, U.K.) for his helpful discussion in the design of the caspase-1 inhibitor experiments (Chapter 7)

Christine Farquhar, (Institute for Animal Health, Edinburgh, UK) for provision of the PrP-specific antiserum 1B3.

I would like to take this opportunity to thank some of closest friends at NPU because without their laughter and encouragement I might have been sentenced to 10 years by now.

Caroline Rafferty- You have been such a support and inspiration to me. Always there to pick up the pieces.

Nicola Gentles- You were there through my undergraduate degree and you were brave enough to stick by me through my PhD. You are a Star!!

Gary Clarke- Everyone needs a small boy to verbally abuse. God bless you.

Dawn Drummond + Tricia Hart- Both of you provided laughter and support in so many ways.

Finally I would like to thank my family, **Mum, Dad, Daniel, Mariea, Kerry, Michael, Jillian, Tailor**. They were always there to remind me that my dreams could be my reality. Finally I thank my **Papa** a very special man.

C

Contents Page

Abstract

Transmissible spongiform encephalopathies (TSEs) are a group of sub-acute infectious neurodegenerative diseases that are characterized by the accumulation in affected tissues of PrP^{Sc}, an abnormal isoform of the host prion protein (PrP^{C}). After peripheral exposure, TSE infectivity and PrP^{Sc} usually accumulate in lymphoid tissues prior to neuroinvasion. Following either oral or intra-peritoneal inoculation follicular dendritic cells (FDCs) are critical for the accumulation of TSE agents within lymphoid tissues and their subsequent neuroinvasion. Studies in mice have shown that exposure through scarified skin is an effective means of transmitting the scrapie agent. Following inoculation via the skin a functional immune system is critical for the transmission of the scrapie agent to the brain as severe combined immunodeficiency (SCID) mice are refractory to infection. However, until now it was not known what components of the immune system are important for scrapie after transmission via the skin. Experiments in this thesis were designed to answer the following questions: firstly does the scrapie agent accumulate within lymphoid tissues after transmission via the skin; secondly what cells are critical for the accumulation and replication of the scrapie agent within lymphoid tissues; and finally how does the scrapie agent reach lymphoid tissues. To answer the first question lymphoid tissues were collected at serial time points after inoculation with the scrapie agent and infectivity titres measured by incubation period assays in indicator mice. Experiments demonstrated that scrapie infectivity first accumulates in the draining lymph node after inoculation via the skin and subsequently spreads to other lymphoid tissues. To address the second aim of this thesis two separate approaches were taken; firstly, a chimeric mouse model was used which had a mismatch in PrP status between FDCs and other bone-marrow derived cells within the lymphoid tissues. This experiment demonstrated that PrP^{C}-expressing FDCs are required for the accumulation of the scrapie agent within the spleen and that the PrP^{C} status of bone-marrow derived cells has no effect on scrapie pathogenesis. Secondly, mice were treated with a reagent to dedifferentiate FDCs either prior to or shortly after challenge to determine the role of FDCs in scrapie neuroinvasion. Data presented in this thesis shows that in the absence of FDCs prior to inoculation, disease susceptibility is reduced. Finally it is not known how the scrapie agent is transported from the site of exposure (e.g. the skin) to the draining lymphoid tissue. Previous studies have suggested that dendritic cells are capable of acquiring and transporting PrP^{Sc} from the gut lumen to the gut associated lymphoid tissues. Langerhans cells (LCs) are a sub-population of migratory dendritic cells which reside in the epidermis and migrate to the draining lymph node following antigen encounter. Thus, LCs were considered a potential candidate for the transportation of the scrapie agent from the skin to the draining lymphoid tissues. To investigate the role of LCs in the active transportation of the scrapie agent from the skin, mouse models were utilised in which active LC migration was blocked. Experiments demonstrated that the early accumulation of the scrapie agent within the draining lymph node and its subsequent neuroinvasion was not impaired in mice with blocked LC migration, however these experiments did not address steady state LC migration, thus a role for this can not be excluded. Although, *in vitro* studies in thesis have suggested that LCs have the potential to acquire and degrade the scrapie agent, suggesting that LCs might be involved in impeding the spread of the scrapie agent after inoculation via the skin.

1

Introduction

1.1 Transmissible spongiform encephalopathies

1.1.1 Historical Background

The transmissible spongiform encephalopathies (TSEs) or "prion diseases" are a group of infectious, fatal neurodegenerative diseases, which affect both animals and humans (Table 1.1). TSEs have previously been described as sub-acute spongiform encephalopathies, slow virus diseases and transmissible dementias. The prototypic TSE disease is scrapie, a naturally occurring disease of sheep and goats, which has been recognized in Europe for over 300 years (McGowan, 1922). Other animal TSE diseases include transmissible mink encephalopathy (TME) (Hartsough and Burger, 1965), chronic wasting diseases (CWD) of mule deer (*Odocoileus hemionus hemionus*) and elk (*Cervus elaphus nelsoni*) (Williams and Young, 1980), and bovine spongiform encephalopathy (BSE) (Wells et al., 1987). Following the discovery of BSE in 1987, feline spongiform encephalopathy of domestic and exotic cats (Wyatt et al., 1991) and spongiform encephalopathies of a number of exotic animals such as kudu and oryx (Jeffrey and Wells, 1988; Kirkwood et al., 1990) were also identified. These TSE diseases are considered to be derived from BSE and transmitted to these species via contaminated feed (Jeffrey and Wells, 1988; Kirkwood et al., 1990; Wyatt et al., 1991). Creutzfeldt Jakob diseases (CJD), Gerstmann-Strausler-Scheinker syndrome (GSS), fatal familial insomnia (FFI) and kuru constitute the major human TSE diseases. These human diseases can be further classified into three etiological categories: sporadic, iatrogenic and familial (Table. 1.1.).

Table 1.1- Transmissible spongiform encephalopathies of animals and humans

TSE	Species	Comments	Reference
Scrapie	Sheep	Prototype TSE first documented by Comber 1772. Transmitted experimentally to goats and rodents. Acquisition unknown	(McGowan, 1922)
Bovine Spongiform Encephalopathy (BSE)	Cattle	Occurred in the U.K 1986 believed to have arisen either sporadically or through ingestion of contaminated MBM.	(Wells et al., 1987)
Feline Spongiform Encephalopathy (FSE)	Domestic and exotic cats	Caused by the same agent which caused BSE, probably due to ingestion of contaminated feed	(Wells et al., 1987)
Exotic TSEs	Ungulates and large wild cats	Caused by the same agent which caused BSE	(Bruce et al., 1997; Wells et al., 1987)
Chronic Wasting Disease	Mule deer and elk	Source unknown, possibly due to the ingestion of contaminated feed	(Williams and Young, 1980)
Transmissible Mink Encephalopathy (TME)	Mink	Present in farmed mink possibly due to ingestion of contaminated offal or through biting during feeding	(Hartsough and Burger, 1965)
Sporadic Creutzfeldt-Jacob disease (CJD)	Human	Occurs possibly due to somatic mutations or spontaneous conversion of PrP^{C} to PrP^{Sc}	(Creutzfeldt,1921 : Jakob 1922)

Variant CJD (vCJD)	Human	Caused by the same agent as BSE, mainly affect young people, probably due to ingestion of contaminated beef	(Bruce et al., 1997; Will et al., 1996)
Iatrogenic CJD (iCJD)	Human	Accidental infection through surgery or treatment with pituitary derived growth hormones	(Duffy et al., 1974)
Familial CJD (fCJD)	Human	Germ-line mutations in *PRNP* gene	(Goldfarb et al., 1992)
Gerstmann Straussler Scheinker Syndrome (GSS)	Human	Germ-line mutations in *PRNP* gene	Gerstmann, 1936
Fatal Familial Insomnia (FFI)	Human	Germ-line mutations in *PRNP* gene	Gambetti, 1992
Kuru	Human	Disease probably acquired through cannibalism of deceased relatives or through handling of infected tissues	(Gajdusek, 1985)

The BSE epidemic, which began in the United Kingdom (UK) in 1985 (Wells et al., 1987), is believed to have arisen from the consumption by cattle of TSE-contaminated protein supplements derived from the carcasses of ruminants (Anderson et al., 1996). The original source of the BSE-agent or the conditions that permitted its emergence in the UK remains unclear (Collee and Bradley, 1997a; Collee and Bradley, 1997b). However, the subsequent appearance of vCJD in 1996 (Will et al., 1996) is considered to be caused by the same TSE strain that caused BSE in cattle (Bruce et al., 1997; Hill et al., 1997), demonstrating that animal TSEs were capable of crossing the species barrier to humans. To date (29th October 2004) there have been 140 deaths from vCJD in the UK (figures from; http://www.cjd.ed.ac.uk/). The epidemic of BSE and the subsequent emergence of vCJD has resulted in intense scientific interest in TSE diseases with the aim of developing both diagnostic and therapeutic strategies against these fatal neurological diseases.

1.1.2 The nature of the TSE agent

The precise nature of the TSE agent is still the subject of debate but currently there are at least three main theories. One theory suggests that the infectious agent is a small conventional virus, with genetic material encoding for its own survival and replication. This hypothesis was proposed on the basis that TSEs are transmissible, display strain specific pathogenesis and the reported presence of viral particles in terminally scrapie-affected brain (Cho, 1976; Cho and Greig, 1975). However, this hypothesis is refuted as experimental evidence demonstrated that the scrapie agent was resistant to viral inactivation treatments such as ultraviolet light, ionizing

radiation, dry heat and chemical treatments (Alper et al., 1966; Brown et al., 1986; Kimberlin et al., 1983c; Somerville et al., 2002; Taylor et al., 1996b).

It was then suggested that it would be thermodynamically feasible for a protein to be the sole component of the infectious agent (Griffith, 1967). Subsequent experiments identified a protease-resistant sialoglycoprotein, which co-purified with infectivity from TSE-affected brains (Bolton et al., 1982; Prusiner, 1982). These data led to the second theory proposed by Stanley Prusiner referred to as the "prion hypothesis" (Prusiner, 1982). The prion hypothesis (derived from the first letters of proteinaceous infectious particle) suggests that PrP^{Sc}, an abnormal, detergent-insoluble, relatively proteinase-resistant isoform of a host glycoprotein PrP^{C} (Meyer et al., 1986) may constitute the major, or sole component of the infectious agent (Prusiner, 1982), replicating in the absence of nucleic acid (Griffith, 1967; Prusiner, 1982). This hypothesis is further supported by recent experimental data, which demonstrated that an abnormally folded fragment of recombinant mouse prion protein causes neurological dysfunction when transmitted to mice, demonstrating for the first time that prions might be infectious (Legname et al., 2004). However, the existence of many different TSE strains is not readily explained by the "prion hypothesis", as it implies that the incoming PrP^{Sc} strain can convert host PrP^{C} regardless of its origin (Bruce, 1993; Bruce and Fraser, 1991). Furthermore, the ability to separate PrP^{Sc} from TSE infectivity is also seen as an argument against the "prion hypothesis" (Somerville and Dunn, 1996; Somerville et al., 2002)

The third theory to be proposed is termed the "virino hypothesis". This hypothesis proposes that agent consists of infection specific informational components that interact with host PrP^C (Dickinson and Fraser, 1979; Dickinson and Outram, 1988) and protects itself from degradation by converting PrP^C to PrP^{Sc} (Farquhar et al., 1998). Such an agent would have the genetic ability to confer different strain-specific characteristics due to the presence of nucleic acid. However, as the agent would not need to encode viral coat proteins the amount of nucleic acid present would be very small and hence might explain why nucleic acid has not been detected in TSE-affected tissues.

1.1.3 The cellular prion protein PrP^C

The *Prnp* gene (*PRNP* in humans, *Prnp* in mice and *PrP* gene in other species) comprises of a single gene in the genomes of rodents, man and ruminants (Oesch et al., 1985; Westway and Prusiner, 1986). *Prnp* encodes a 33-35 kDa sialoglycoprotein consisting of approximately 210 amino acid residues. The PrP^C protein is highly conserved amongst mammals and expressed early during embryogenesis (Manson et al., 1992). The highest level of PrP^C expression is found within the central (CNS) and peripheral (PNS) nervous systems (Oesch et al., 1985) but it is also expressed to varying degrees in the tissues of the periphery (Caughey et al., 1988; Dodelet and Cashmann, 1998; McBride et al., 1992). PrP^C consists of several distinct domains including a N-terminal signal peptidc, a series of at least four Cu^{2+} binding octapeptide repeats and a glycophosphatidylinositol (GPI) moiety that attaches the protein to the plasma membrane (Fig. 1.1). PrP^C contains two sites for N-glycosylation at residues Asn_{181} and Asn_{197} and differential glycosylation of.

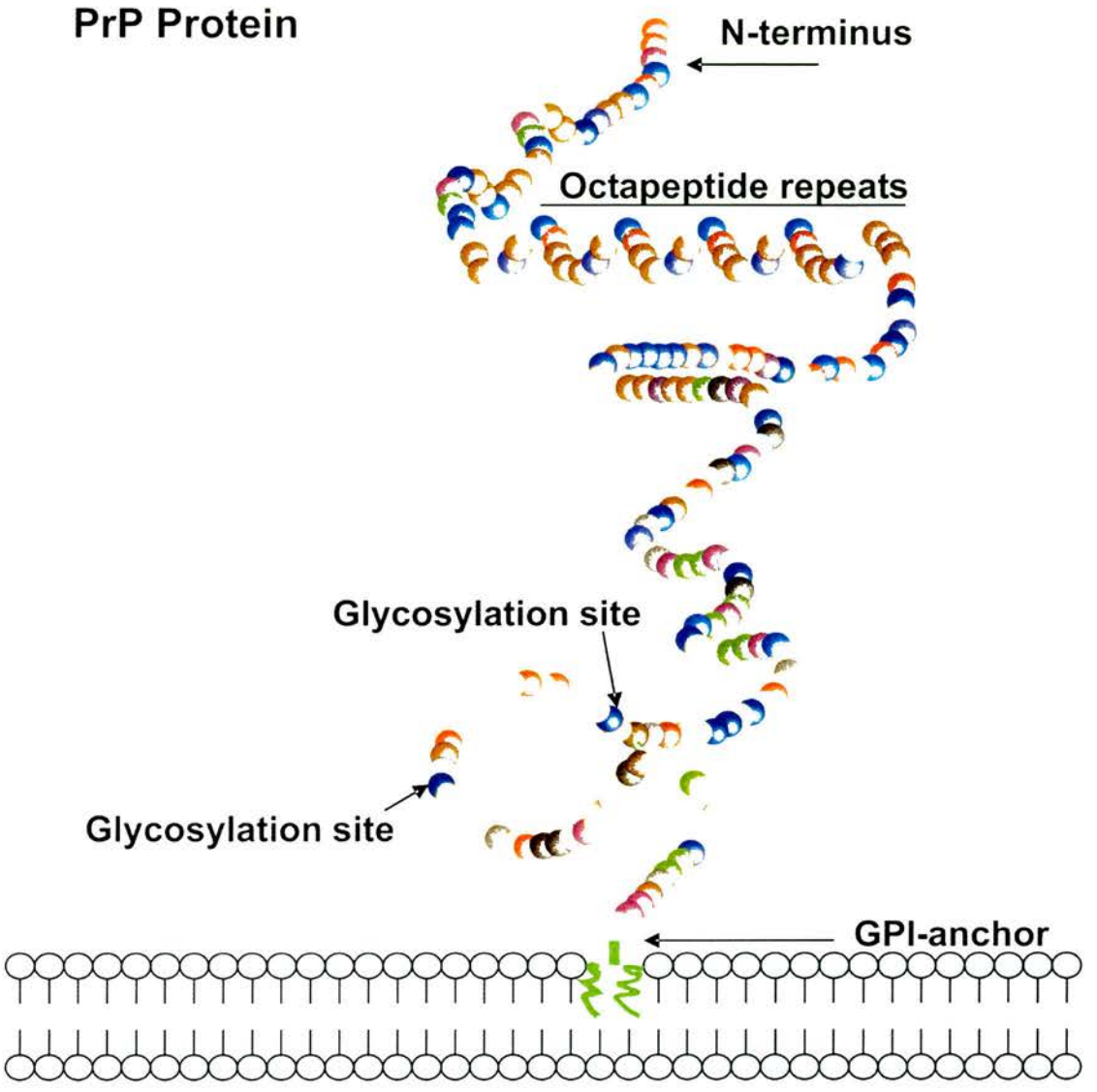


Figure 1.1- A schematic representation of the normal host cellular protein PrP^C. The PrP^C protein consists of a glycosyl-phosphatidyl-inositol (GPI) anchor which attaches it to the cellular membrane of the cell, a series of copper binding octapeptide repeats and two glycosylation sites at residues 181 and 197 (Oesch et al., 1985). Figure originally created by Wilfred Goldmann (NPU, Edinburgh).

these sites results in three cellular glycoforms; unglycosylated, monoglycosylated and diglycosylated (Oesch et al., 1985). The function of PrP^C is unknown, as mice deficient in the *Prnp* gene (*Prnp*$^{-/-}$ mice) display no gross phenotypic changes (Bueler et al., 1992; Manson et al., 1994a). However, there have been reports of subtle abnormalities such as; disturbed circadian rhythms (Tobler et al., 1996) and reduced T-lymphocyte responses (Cashman et al., 1990; Kubosaki et al., 2003; Lewicki et al., 2003; Mabbott et al., 1997). Due to the high expression of PrP^C in the CNS, experimental research has focused on this tissue to try to determine the function of PrP^C. It has been suggested that a major function of PrP^C might be to promote neuronal survival by protecting against apoptosis, as *Prnp*$^{-/-}$ cells are more susceptible to apoptosis following serum deprivation (Bounhar et al., 2001; Kim et al., 2004; Kuwahara et al., 1999; Roucou et al., 2004). Similarly, recent research has shown that cross-linking of PrP^C *in vivo* by treatment with PrP-specific monoclonal antibodies triggers extensive apoptosis of neurons (Solforosi et al., 2004). Alternatively, the ability of PrP^C to bind copper (Cu) and block Cu toxicity to neurons (Brown, 2004; Brown et al., 1997a; Brown et al., 1997b; Millhauser, 2004) coupled with increase in oxidative stress markers in the brains of *Prnp*$^{-/-}$ mice (Milhavet et al., 2000; Wong et al., 2001), suggests that PrP^C might also play a role in protecting neurons against physiological stress. Taken together these data suggest that PrP^C is most likely to have a neuroprotective function within the CNS.

1.1.4 PrP and its role in TSE pathogenesis

Prnp$^{-/-}$ mice are refractory to TSE disease demonstrating that expression of host PrP^C is critical for TSE disease (Bueler et al., 1993; Manson et al., 1994a). Furthermore,

experiments where brain tissue from PrP-expressing wild-type mice was grafted into the brains of *Prnp*$^{-/-}$ mice have shown that TSE infection developed and spread only within the PrP-expressing graft tissue (Brandner et al., 1996). The development of TSE diseases is also affected by PrP gene dosage (Manson et al., 1994b). PrP heterozygote mice (*Prnp*$^{+/-}$) with only one functioning *Prnp* allele have a longer disease incubation period than wild-type mice (*Prnp*$^{+/+}$) with two functioning *Prnp* alleles (Manson et al., 1994b). However, the pattern and level of deposition of PrP^{Sc} is the same for both mouse models at the terminal stage of the disease, demonstrating that the accumulation of PrP^{Sc} is not limited by the expression of PrP^{C} by the host (Manson et al., 1994b).

One factor that influences survival time in TSE-affected mice is the *Sinc* (scrapie incubation) gene (Dickinson et al., 1968). *Sinc* has two alleles *s7* and *p7* that result in short or prolonged disease incubation periods with the ME7 strain of mouse-passaged scrapie, respectively (Dickinson and MacKay, 1961; Dickinson and Meikle, 1971; Dickinson et al., 1968). Sinc also has a major influence on the incubation period of other TSE strains but the direction and magnitude of the effect differs between strains (Bruce and Fraser, 1991). Subsequent research has shown that *Sinc* gene is congruent to the mouse *Prnp* gene and the dimorphisms at codon 108 and codons 189 are now referred to as *Prnp*^{a} (Leu-108, Thr-189; *Sinc*^{s7}) and *Prnp*^{b} (Phe-108, Val-189; *Sinc*^{p7}) (Moore et al., 1998). However, a recent study of inbred mouse lines has identified a third allele termed *Prnp*^{c} (Phe-108, Thr-189) that has been shown to influence the disease incubation period (Lloyd et al., 2004). Classically, *Sip* the ovine homolog of *Sinc/Prnp* performs the same function in sheep

and was originally described as having two alleles *sA* and *pA* (Dickinson and Outram, 1988; Hunter et al., 1992). However, several polymorphisms within the ovine *PrP* gene have been identified that are related to disease pathogenesis, notably at codons 136, 154 and 171. These polymorphisms occur in different allelic combinations which can confer either susceptibility or resistances to scrapie infection dependent on the combination of alleles and the bred of sheep (Goldmann et al., 1991; Goldmann et al., 1994; Hunter, 1998). Similarly, polymorphisms in the *PRNP* gene in humans are linked to disease susceptibility (Prusiner, 1998). For example, a change at codon 129 is linked to sCJD (Goldfarb et al., 1992), vCJD (Ironside, 2000) and kuru (Lee et al., 2001) susceptibility. Similarly, mutation at codon 102 is linked to susceptibility to GSS (Hsiao et al., 1989). However, factors other than polymorphisms of the *Prnp* gene can influence TSE pathogenesis such as the route of inoculation, the dose of the inoculum and the strain of the agent (Farquhar et al., 1994).

One of the most significant scientific discoveries within TSE research was the identification of the protein termed PrP^{Sc}. Initial ultra-structural studies of scrapie infected brains demonstrated the presence of abnormal scrapie associated fibrils (SAF) (Mertz et al., 1981). It was subsequent research by Prusiner *et al* that demonstrated that SAF contains an abnormal protein, termed PrP^{Sc}, which also co-purifies with scrapie infectivity (Prusiner, 1982). The prion hypothesis (section 1.1.2) proposed that PrP^{Sc} is the sole component of the infectious agent (Prusiner, 1982), replicating in the absence of nucleic acid (Griffith, 1967; Prusiner, 1982) by

structurally converting the host cellular protein PrP^{C} into the pathogenic isoform, PrP^{Sc} (Prusiner, 1991).

To date approximately 20 phenotypically distinct scrapie strains have been identified through serial passage in lines of inbred mice. The finding that there are many distinct strains of scrapie is not readily explained by the prion hypothesis as it implies that the incoming PrP^{Sc} strain can convert host PrP^{C} regardless of its origin into the same abnormal conformation (Bruce, 1993; Bruce and Fraser, 1991). It has been proposed that differing TSE strains are due to differing conforms of PrP^{Sc} (Prusiner, 1991), or to differences in PrP^{Sc} glycosylation patterns (Ermonval et al., 2003). Although PrP^{Sc} was initially shown to co-purify with scrapie infectivity (Bolton et al., 1982; Prusiner, 1982) experimental data has shown that in some murine TSE models the level of PrP^{Sc} accumulation does not always correlate with the level of scrapie infectivity in the affected tissue (Barron et al., 2001; Lasmezas et al., 1997; Manson et al., 1999; Somerville and Dunn, 1996). However, the murine ME7 scrapie model used in this study has been well characterized at the Neuropathogenesis Unit (NPU), Edinburgh, and in this model there is distinct correlation between the increasing presence of PrP^{Sc} in affected-tissues and the presence of scrapie infectivity (Brown et al., 1999; Farquhar et al., 1994; Mabbott et al., 2002; Mabbott et al., 2003). Thus, PrP^{Sc} is considered a good disease-specific marker for scrapie infection in this study.

1.1.5 Distinguishing between PrP^{C} and PrP^{Sc}

PrP^{Sc} contains a proteinase resistant core with a molecular weight of 27-30 kDa and is distinguishable from the normal host cellular protein PrP^{C} by proteinase K (PK) digestion. Immunoblots of uninfected and TSE affected tissues have shown that while $PrP^{33\text{-}35kDa}$ from an uninfected animal is fully hydrolysised by PK digestion, $PrP^{33\text{-}35kDa}$ from TSE-affected tissues is only partially degraded, leaving the proteinase resistant core protein $PrP^{20\text{-}30kDa}$ (Oesch et al., 1985). TSE-affected tissues can be identified by PK digestion and immunoblotting. Following immunoblotting a typical three-banded pattern between molecular mass values of 20-30 kDa, representing the unglycosylated, monoglycosylated and diglycosylated isomers of PrP (in order of increasing molecular mass) is seen demonstrating the presence of disease-specific PrP^{Sc}. PrP^{Sc} is also distinguishable from PrP^{C} based on its conformation. Infrared and circular dichroism experiments have shown that PrP^{C} consists of approximately 43% α-helix and 3% β-sheets, while PrP^{Sc} consists of approximately 34% α-helix and 43% β-sheets (Pan et al., 1993). At present there are no commercially available antibodies which can specifically distinguish between PrP^{C} and PrP^{Sc}, although Paramithiotis and colleagues have recently described an antibody which specifically recognises PrP^{Sc} (Paramithiotis et al., 2003). The absence of antibodies that can specifically distinguish between PrP^{C} and PrP^{Sc} can be problematic for experimental techniques that do not allow for PK digestion. However, analysis of the level of deposition and localisation of PrP^{Sc} in TSE affected tissues (Beekes and McBride, 1998; Bruce et al., 1989) and use of serial tissue sections for PK negative and PK positive experimental protocols (Schulz-Schaeffer et al., 2000) allows this difficulty to be circumvented.

1.1.6 Scrapie

Scrapie is a naturally occurring TSE disease of sheep and goats, which has also been referred to as la tremblante (French for trembling), traberkrankheit (German for trotting disease), and rida (Icelandic for ataxia or tremor) (Detwiler et al., 2000). Scrapie is present in the UK and in most sheep producing regions worldwide, with the notable exceptions of Australia and New Zealand, which have not reported the presence of scrapie.

Initially scrapie was believed to be an autosomally recessive genetic disease which was not naturally infectious (McGowan, 1922). However, this hypothesis was challenged when scrapie was accidentally transmitted to sheep via a vaccination for louping ill disease derived from a suspension of brain and spleen homogenate, which was unknowingly contaminated with scrapie (Gordon, 1946). In 1936, scrapie was experimentally demonstrated to be transmissible by inoculation between sheep and goats (Cuille and Chelle, 1936). Subsequently, research highlighted the similarities between scrapie and other TSEs at the neuropathological, clinical and epidemiological levels (Hadlow, 1959). From the early 1960s scrapie has been experimentally transmitted to laboratory rodent models (Chandler, 1961; Chandler, 1963; Marsh and Hanson, 1971) and has been shown to be transmissible both horizontally among flocks of sheep and vertically from ewe to lamb (Brotherston et al., 1968; Foster et al., 1996; Haralambiev et al., 1973). The route of natural scrapie transmission is unknown, but 2-5 years after the animal is infected clinical signs of scrapie infection appear which include tremors of the head and neck, scratching,

rubbing, lip smacking and gait abnormalities, with death being inevitable (McGowan, 1922).

1.1.7 TSE strain identification and neuropathological characterisation in rodent models

To date over 20 different scrapie strains have been identified through serial passage, mainly in lines of inbred mice. Strains are identified according to the duration of their highly reproducible incubation periods and their characteristic neuropathlogy. When a serially-passaged scrapie strain is injected intra-cerebrally at a high dose into a group of inbred mice, the mean disease incubation period is highly reproducible, generally giving a standard error of less than 2% of the mean (Dickinson and Meikle, 1971; Dickinson et al., 1968). In tissue sections from the brain of a scrapie-affected animal tiny holes in the neuropil of both the grey and white matter, referred to as vacuolation are present (Fig 1.2). Vacuolation differs in severity and distribution according to the strain of scrapie agent; however, host genotype and route of challenge can also affect the vacuolation pattern (Fraser, 1979; Fraser and Dickinson, 1973). If strain of agent, host genotype and route of challenge are kept constant then scrapie strains can also be identified based on their quantitative pattern of vacuolar pathology (Fraser, 1979; Fraser and Dickinson, 1973). More recently, a technique called glycoform analysis has been used to distinguish TSE strains (Collinge et al., 1996; Parchi et al., 1995; Somerville, 1999; Somerville et al., 1997b). This technique distinguishes TSE strains based on the intensity and migration of their PrP glycoform banding pattern obtained by gel electrophoresis.

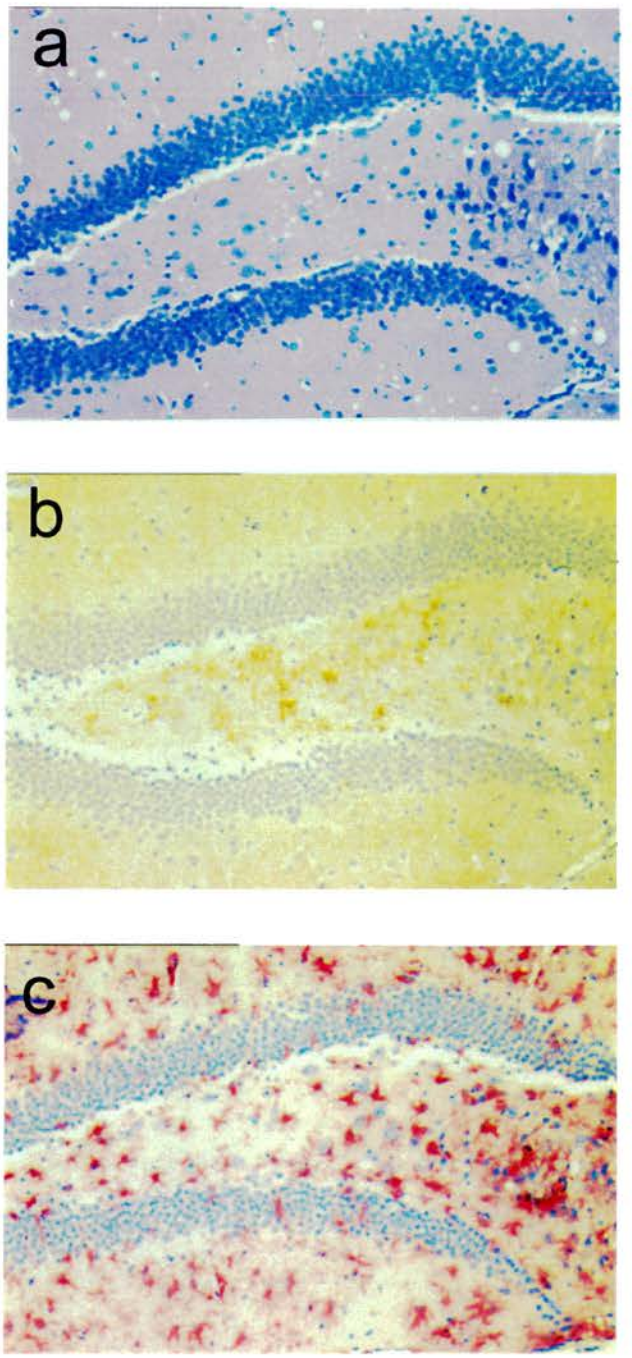


Figure 1.2- Classical hallmarks of TSE disease within the brain are vacuolation of the neuropil (a), deposition of the disease-specific protein PrP as determined by immunolabelling with the PrP-specific monoclonal antiserum 6H4 (b; brown) and glial cell activation as determined by immunolabelling with the monoclonal antiserum GFAP (c; red). All sections were counterstained with haematoxylin to distinguish cell nuclei. Original magnification X200

There are four neuropathological hallmarks of TSE infection in rodent models (Fig. 1.2), although the severity of each varies according to the strain. These features include glial cell activation resulting in an increase in the number and size of astrocytes (Bruce et al., 1994; Deidrich et al., 1991) and microglial cells (Williams et al., 1997), neuronal loss (Fraser, 2002), characteristic vacuolar pathology (Fraser, 1979; Fraser and Dickinson, 1973) and PrP^{Sc} deposition (Bendheim et al., 1984; DeArmond et al., 1985). Glial cell activation can occur as a result of many neurological diseases, however, the presence of vacuolation and PrP^{Sc} deposition are key to the identification of TSE infection (Fig 1.2).

1.2 Peripheral pathogenesis of TSEs

1.2.1 Routes of exposure

Natural TSEs are usually acquired by peripheral exposure. The possibility of oral acquisition was first highlighted during a study of the Fore people of Papua New Guinea (Gajdusek, 1985). In this study, the transmission of kuru was most likely associated with the ritualistic practice of cannibalizing brains of deceased relatives (Gajdusek, 1985). Data also suggests the consumption of BSE-contaminated feed by cattle and the subsequent consumption of BSE-contaminated meat by humans is responsible for the emergence of BSE (Wilesmith et al., 1991) and vCJD respectively (Bruce et al., 1997; Hill et al., 1997). The natural acquisition route of scrapie is unknown, however it is likely to be acquired orally as PrP^{Sc} was first detected in the Peyer's patches and GALT of naturally infected sheep (Andreoletti et al., 2000; Heggebo et al., 2000).

Although oral acquisition is considered the main route of natural exposure to TSE agents, other routes of peripheral exposure have been identified. Sporadic CJD in humans has been transmitted iatrogenically through transplantation of CJD contaminated tissues or pituitary-derived hormones (Duffy et al., 1974). Concerns therefore also exist that vCJD might be transmitted in a similar manner (Brown et al., 1992; Hilton et al., 2004). The BSE agent has recently been shown to be transmissible by blood transfusions in sheep (Houston et al., 2000; Hunter, 2003; Hunter and Houston, 2002), as has the vCJD agent in humans (Llewelyn et al., 2004; Pincock, 2004). Studies in mice have shown that transmission via the skin is also an effective means of establishing TSE infection (Taylor et al., 1996a), suggesting that skin lesions might be a route of natural exposure.

1.2.2 The lymphoreticular system in TSE pathogenesis

Following peripheral exposure, infectivity and PrP^{Sc} usually accumulate within the lymphoreticular system (LRS) prior to neuroinvasion (Eklund et al., 1967). The role of the LRS was first demonstrated after subcutaneous (s.c.) inoculation of mice with the Chandler scrapie strain (Eklund et al., 1967). Assays of various tissues at sequential time points post-inoculation demonstrated that scrapie infectivity was detectable within the spleen and peripheral lymph nodes at 4 weeks post-inoculation, where it accumulated to high levels prior to detection within the CNS (Eklund et al., 1967).

The role of the spleen in peripheral scrapie pathogenesis was further demonstrated by studies which showed that genetic asplenia or splenectomy either prior to or shortly

after peripheral exposure to the scrapie agent significantly extended the survival time of experimentally inoculated rodents (Clarke and Haig, 1971; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978). However, the presence of the spleen is not obligatory in the establishment of scrapie infection following peripheral exposure as splenectomy does not affect disease susceptibility, suggesting that other tissues are able to sustain replication in its absence. Furthermore, splenectomy of mice before s.c inoculation with the scrapie agent has no affect on the disease incubation period, suggesting that the role of the spleen in scrapie pathogenesis is dependent on the route of exposure (Fraser et al., 1992; Kimberlin and Walker, 1989a).

The use of severe combined immunodeficiency (SCID) mice, which lack mature B and T-lymphocytes (Bosma et al., 1983 288), has further demonstrated the importance of the LRS in scrapie pathogenesis. SCID mice are resistant to scrapie infection following peripheral exposure to moderate or low doses of scrapie inoculum (Fraser et al., 1996; O'Rourke et al., 1994). Grafting of SCID mice with bone-marrow from immunocompetent donors restores susceptibility to scrapie infection and replication within the spleen (Fraser et al., 1996; Lasmezas et al., 1996; O'Rourke et al., 1994). Furthermore, pre-treatment of mice with pharmacological agents which either stimulate or depress the immune system prior to inoculation with the scrapie agent, results in either a shortening or lengthening of the disease incubation period, respectively (Dickinson et al., 1978; Farquhar and Dickinson, 1986; Mabbott et al., 2001; Outram et al., 1974; Sethi et al., 2002).

The involvement of the LRS in TSE pathogenesis was believed to be dependent on host-strain interactions as BSE in cattle (Somerville et al., 1997a) and sporadic CJD in humans (Hill et al., 1999) appear to be confined to the nervous tissues. However, recent research has demonstrated the presence of PrP^{Sc} in the spleens of some Swiss sporadic CJD patients at the terminal stage of the disease, although its relevance to disease pathogenesis is not known (Glatzel et al., 2003). At present the targeting of the LRS prior to neuroinvasion has been demonstrated in vCJD patients (Hilton et al., 1998), CWD in deer and elk (Sigurdson et al., 1999), sheep with natural scrapie (van Keulen et al., 1996) and rodents experimentally inoculated with scrapie (McBride et al., 1992).

Extensive studies have been conducted to determine the contribution of different cell types within the LRS to peripheral scrapie pathogenesis (Mabbott and Bruce, 2001; Mabbott et al., 1998). The role of some of these cell types is reviewed below.

1.2.3 T-lymphocytes

T-lymphocytes are unlikely to be involved in peripheral scrapie pathogenesis as depletion of this cell population either by thymectomy or sub-lethal whole body γ-irradiation has no affect on the disease incubation period following peripheral inoculation (Fraser and Dickinson, 1978; Fraser and Farquhar, 1987). Similarly studies using transgeneic or immunodeficient mice which lack functional T-lymphocytes ($CD4^{-/-}$, $CD8^{-/-}$, β_2-$\mu^{-/-}$, $TCR\alpha^{-/-}$, $perforin^{-/-}$, SCID, *Rag-1*$^{-/-}$ or *Rag-2*$^{-/-}$ mice) have shown that deficiencies in T-lymphocytes alone have no affect on the accumulation of infectivity within the LRS or its subsequent neuroinvasion(Fraser et

al., 1996; Klein et al., 1997; Klein et al., 1998). In addition, the expression of PrP^C only on T-lymphocytes is insufficient to sustain the replication of the scrapie agent in the LRS (Raeber et al., 1999a), suggesting that PrP expression is not the only requirement for sustaining agent replication. Recent research has shown that a combined deficiency of $CD4^+$ and $CD8^+$ cell populations prior to either i.c. or i.p. inoculation with scrapie strain RML results in a significantly longer disease incubation period (Lewicki et al., 2003). As the effects are observed after both i.c. and i.p. inoculation this suggests that disease pathogenesis in the CNS, not the LRS is affected in the absence of both CD4 and CD8 cell populations (Lewicki et al., 2003). Furthermore, as no affect on disease susceptibility was observed in the study this suggests that any role T-lymphocytes might play in scrapie pathogenesis in this model is unlikely to be significant due to full susceptibility of the above model to scrapie infection (Lewicki et al., 2003).

1.2.4 Natural killer cells

The role of natural killer (NK) cells in peripheral scrapie pathogenesis has not been directly addressed. However, studies investigating the role of B and T-lymphocytes have indirectly suggested that NK cells are unlikely to play a key role in scrapie pathogenesis within the LRS (Klein et al., 1997). The role of NK cells in innate immunity is to directly lyse virus-infected cells and tumour cells and to secrete various cytokines such as interferon-γ to initiate an immune response (Roitt, 1994). Studies have shown that the absence of NK effector molecules such as perforin (perforin$^{-/-}$ mice) involved in cell lysis and interferon receptors (*Arg*$^{-/-}$ mice) have no

effect on scrapie pathogenesis (Klein et al., 1997) suggesting NK cells are not involved in the lysis of scrapie infected cells.

1.2.5 B-lymphocytes

Following peripheral inoculation of SCID (Bosma et al., 1983), *Rag-1*$^{-/-}$ (Mombaerts et al., 1992), *Rag-2*$^{-/-}$ (Shinkai et al., 1992) *Arg*$^{-/-}$ (Huang, 1993), and μMT (Kitamura et al., 1991) mice which all lack mature B and T-lymphocytes, accumulation of infectivity in the spleen is blocked and subsequent neuroinvasion impaired (Brown et al., 1997c; Fraser et al., 1996; Klein et al., 1997; O'Rourke et al., 1994). As the role of T-lymphocytes had been previously determined (section 1.2.3) these data led to the suggestion by Klein *et al* that B-lymphocytes might play a key role in scrapie pathogenesis (Klein et al., 1997), as deficiencies in these lymphocytes impaired scrapie neuroinvasion. However, research, which reconstituted immunodeficient mice with foetal liver cells from either $PrP^{-/-}$ deficient or $PrP^{+/+}$ expressing mice (Brown et al., 1999; Klein et al., 1998), demonstrated, that susceptibility to scrapie infection was restored in the presence of PrP-deficient B-lymphocytes so long as PrP^{C} was expressed by stromal derived cells (Brown et al., 1999; Klein et al., 1998). These data suggest that a cell population whose maturation depends on B-lymphocytes is critical to scrapie pathogenesis following peripheral exposure.

B-lymphocytes produce important cytokine signals that mature and maintain follicular dendritic cells (FDCs) within the germinal centres (Chaplin and Fu, 1998; Kosco-Vilbois et al., 1997). Therefore mice deficient in mature B-lymphocytes are also indirectly deficient in mature FDCs. Subsequent studies were therefore required

to determine whether B-lymphocytes or FDCs were the key cell type involved in sustaining the replication of the scrapie agent within the LRS.

1.2.6 Follicular dendritic cells (FDCs)

FDCs are considered to be derived from undifferentiated stromal precursor cells within lymphoid tissues (Tew et al., 1999). They are restricted to the light zones of the germinal centres within lymphoid follicles where they function to trap and retain antigen on their surface in the form of immune complexes for long term presentation to B-lymphocytes (Tew et al., 1997). B-lymphocytes compete for and bind antigens on FDCs and emerge as either antibody forming cells, producing high affinity antibodies or as memory B-cells with high affinity receptors (Tew et al., 1997).

A number of biological properties of FDCs correspond with the experimental evidence indicating that this cell type is likely to be involved in peripheral scrapie pathogenesis. Studies by Clarke & Kimberlin have shown that the level of scrapie infectivity present in the stromal compartments of the LRS, in which FDCs are located, was on average ten times higher than that in the white pulp where B-lymphocytes are located (Clarke and Kimberlin, 1984). Sub-lethal whole body γ-irradiation depletes actively dividing lymphocytes and monocytes (Fulop and Phillips, 1986; Phillips and Fulop, 1989). When this treatment was administered to mice, prior to or shortly after peripheral inoculation, no affect on scrapie pathogenesis was detected (Fraser and Farquhar, 1987). This suggests the cell type involved in scrapie pathogenesis within the lymphoid tissues is a radioresistant,

mitotically inactive cell, of stromal origin, criteria that FDCs fulfil (Tew et al., 1999; Tew et al., 1990; Tew et al., 1997).

Subsequent studies have demonstrated that within weeks of peripheral infection with the scrapie agent, disease-specific PrP immunolabelling is detectable on the surface and within the extracellular spaces of FDC processes (Jeffrey et al., 2000b; Mabbott et al., 2002). PrP^{Sc} has also been detected in association with FDCs in the LRS; of mice infected with scrapie (Mabbott et al., 2000b) or CJD (Kitamoto et al., 1991), sheep with natural scrapie (van Keulen et al., 1996), mule deer fawns with CWD (Sigurdson et al., 1999) and patients with vCJD (Hill et al., 1999).

Immunohistochemical labelling has suggested that FDCs in uninfected mice express high levels of PrP^{C} (Brown et al., 1999; McBride et al., 1992). The expression of PrP^{C} by FDCs has been shown to be critical for the accumulation of the scrapie agent within the LRS (Brown et al., 1999; Klein et al., 1998; Raeber et al., 1999b). In the study by Brown and colleagues two mouse lines were produced by crossing the *scid* mutation into either PrP deficient mice ($Prnp^{-/-}$) or PrP expressing wild-type mice ($Prnp^{+/+}$) (Brown et al., 1999). These SCID mice were then reconstituted with either bone-marrow from $Prnp^{+/+}$ expressing or $Prnp^{-/-}$ deficient mice. As a consequence, the lymphocytes with a PrP status of the graft origin would induce the maturation of FDC precursor cells with a PrP status of recipient origin. Using these chimeric models immunolabelling for PrP in the spleen could only been detected on FDCs when the FDCs themselves were carrying a $Prnp^{+/+}$ gene and was independent of the PrP status of the surrounding lymphocyte populations. This study demonstrated that

FDCs express PrP^C and do not acquire it from neighbouring lymphocytes (Brown et al., 1999). Furthermore, when these models were peripherally inoculated with the ME7 scrapie strain, infectivity was only detected within spleens which contained PrP^C-expressing FDCs (Brown et al., 1999). None was detectable within the spleens of animals which expressed PrP^C only on lymphocyte populations, demonstrating that PrP^C-expressing FDCs are critical for substaining scrapie infection within the LRS (Brown et al., 1999). However, current evidence suggests that different TSE strains might differ in their targeting of different cell populations within the immune system. For example, efficient replication of the RML scrapie isolate, unlike ME7 scrapie strain might require both PrP^C-expressing FDCs and lymphocytes (Kaeser et al., 2001; Klein et al., 1998).

However, research has demonstrated that the expression of PrP^C is not the only cellular requirement for TSE infection (Lotscher et al., 2003; Montrasio et al., 2001; Raeber et al., 1999a). Studies using transgenic *Prnp*$^{-/-}$ mice over-expressing PrP^C on either B or T-lymphocytes alone (Montrasio et al., 2001; Raeber et al., 1999a) have shown that PrP^{Sc} does not accumulate within the LRS following peripheral inoculation. Furthermore, high levels of PrP^C are expressed by the capsule and trabeculae of the spleen (Lotscher et al., 2003) in both wild-type and immunodeficient *Rag-1*$^{-/-}$ mice (Mombaerts et al., 1992). As *Rag-1*$^{-/-}$ mice are refractory to scrapie infection (Klein et al., 1997) expression of PrP^C by the capsule and trabeculae clearly are not critical for scrapie pathogenesis (Lotscher et al., 2003).

As PrP^C expression is unlikely to be the only cellular requirement for sustaining the replication of the scrapie agent, further studies were conducted to identify other biological properties of FDCs which makes them suitable cellular targets for scrapie infection (Klein et al., 2001; Lotscher et al., 2003; Mabbott et al., 2001). The function of FDCs is to trap and retain antigens in the form of immune complexes (Nossal et al., 1968a; Nossal et al., 1968b). Deficiencies in either circulating antibodies or Fc-receptors (FcγR, FcγRII or FcγRIII) have no effect on TSE pathogenesis (Klein et al., 2001). However, studies have suggested that complement components and cellular complement receptors may play a role in the localization and retention of PrP^{Sc} to FDCs (Klein et al., 2001; Mabbott et al., 2001). Genetic deficiency in C3 (Klein et al., 2001) or temporary depletion of C3 (Mabbott et al., 2001) impairs PrP^{Sc} accumulation in the spleen and significantly prolongs the incubation period after peripheral challenge with the scrapie agent. Furthermore, mice lacking the complement components C3, C2 or factor B (Taylor, 1998) have significantly prolonged incubation periods following peripheral exposure to the scrapie agent (Klein et al., 2001; Mabbott et al., 2001). However, a greater extension of the disease incubation period was observed in mice deficient in C1q ($C1qa^{-/-}$; (Botto, 1998)) suggesting a possible role for C1q alone (Klein et al., 2001; Mabbott et al., 2001). In addition, recent research has suggested that C1q may induce the up-regulation of PrP^C by FDCs, which might enhance susceptibility to scrapie infection (Lotscher et al., 2003).

The contribution of FDCs to scrapie accumulation within the LRS and its subsequent neuroinvasion was further investigated by altering the immunological relationship

that exists between FDCs and B-lymphocytes. Two key cytokines produced by B-lymphocytes, which maintain FDCs in their dedifferentiated state are tumour necrosis factor α (TNF-α), which signals through TNF-receptor 1/p55 (TNF-R1) (Tkachuk et al., 1998), and membrane bound lymphotoxin (LTα/β), whose signal is mediated through the LTβ receptor (LTβR) (Endres et al., 1999). Each of these receptors is expressed on the surface of FDCs and/or their precursors (Tew et al., 1997). In contrast a deficiency in interleukin-6 (IL-$6^{-/-}$), a soluble cytokine produced by FDCs which signals the maturation and differentiation of B-lymphocytes in germinal centres, impairs B lymphocyte development and thus impairs germinal centre formation, however, FDC networks are maintained.

Research has demonstrated that deficiencies in germinal centres alone does not affect peripheral scrapie pathogenesis, as peripherally challenged IL-$6^{-/-}$ mice accumulate infectivity and PrP^{Sc} within their spleens to the same extent as wild-type mice, and no differences in incubation periods were observed (Mabbott et al., 2000b). In contrast mice deficient in either TNFα (Pasparakis et al., 1996) or TNF-R1 (Matsumoto et al., 1996b) lack mature FDCs within lymphoid tissues, although the presence and function of lymphocytes are maintained. Studies have shown that TNF$\alpha^{-/-}$ knockout mice are less susceptible to peripheral challenge with the ME7 scrapie strain than wild-type mice and fail to accumulate infectivity or PrP^{Sc} in their spleens (Mabbott et al., 2002; Mabbott et al., 2000b). Similarly previous research has shown that LT$\alpha^{-/-}$, LT$\beta^{-/-}$ and LT$\beta R^{-/-}$ mice, which all lack mature FDC networks (Futterer et al., 1998; Koni et al., 1997; Matsumoto et al., 1996a), fail to accumulate scrapie in the LRS and are less susceptible to peripheral inoculation with the RML

scrapie isolate (Prinz et al., 2002). Similarly the, temporary dedifferentiation of FDCs by treatment with the LTβR immunoglobulin-fusion protein (LTβR-Ig; (Force et al., 1995), which blocks the LTβR-signaling pathway (Mackay and Browning, 1998), inhibits the early accumulation of PrP^{Sc} and scrapie infectivity in the LRS and reduces disease susceptibility (Mabbott et al., 2000a; Mabbott et al., 2003; Montrasio et al., 2000). Taken together the above studies demonstrated that mature FDCs are critical for scrapie pathogenesis following peripheral exposure.

However, studies have demonstrated that neuroinvasion of the scrapie agent can occur in the absence of FDCs. Studies using the RML scrapie isolate have shown that TNFR-$1^{-/-}$, TNFR-$2^{-/-}$ and TNF-$\alpha^{-/-}$ mice are as susceptible as wild-type mice to scrapie infection (Klein et al., 1997; Prinz et al., 2002). These data suggest that in some circumstances neuroinvasion of the RML scrapie isolate might occur via a FDC-independent route. However, in some studies large doses of inoculum were used which might directly infect peripherally nerves and thus, bypass replication within the LRS (Fraser et al., 1996; Klein et al., 1998; Mabbott et al., 2000b; Oldstone et al., 2002). In addition TNFR and TNF-α play a key role in mediating macrophage function (Ma, 2001) therefore, it is likely that in their absence macrophages will be impaired in the clearance of the agent. Further studies are required to determine whether different scrapie strains utilise different cell population for accumulation and replication within the LRS.

1.2.7 Macrophages

Both *in vitro* and *in vivo* studies have demonstrated that macrophages are capable of sequestering and degrading scrapie infectivity (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982). Depletion of this cell population, prior to or shortly after, peripheral inoculation results in increased accumulation of infectivity and PrP^{Sc} in the spleen and shortens the disease incubation period (Beringue et al., 2000b). Furthermore, during scrapie infection intra-lysosomal PrP^{Sc} accumulations have been detected within tingible body macrophages in the spleens of rodents experimentally infected with scrapie (Jeffrey et al., 2000b), naturally scrapie infected sheep (Hermann et al., 2003; Jeffrey et al., 2000a) and naturally CWD infected deer (Sigurdson et al., 2002). Thus, tingible body macrophages might acquire PrP^{Sc} by either phagocytosis of FDC processes or from within the extracellular spaces between FDC processes (Hermann et al., 2003; Jeffrey et al., 2000a; Sigurdson et al., 2002). Recent research by Prinz *et al* has suggested that in some circumstances, such as in $TNF\alpha^{-/-}$ deficient mice, macrophages might also be involved in replication of the scrapie agent within the LRS following exposure to a large dose of scrapie inoculum (Prinz et al., 2002).

1.2.8 The immune response to TSE infection

Despite the accumulation of high titres of TSE infectivity within the LRS of infected animals, TSE infection does not appear to induce an immune response in the host. For example, no TSE-specific detectable humoral immune response has been observed (Chandler, 1959; Clarke and Haig, 1966; Marsh et al., 1970; Pattison et al., 1964; Porter et al., 1973). Furthermore, deficiencies in either circulating antibodies

or Fc-receptors (FcγR, FcγRII or FcγRIII) have no effect on TSE pathogenesis (Klein et al., 2001). Similarly, there is no induction of cell-mediated immunity during scrapie infection (Kingsbury et al., 1981). In support of these data, studies using transgenic or immunodeficient mice, which lack functional T-lymphocytes ($CD4^{-/-}$, $CD8^{-/-}$, β_2-$\mu^{-/-}$, $TCR\alpha^{-/-}$ or $perforin^{-/-}$ mice) (Klein et al., 1997; Klein et al., 1998) have shown that the absence of functional T-lymphocytes has no effect on disease pathogenesis. The lack of a immune response to the TSE agent is not due to immunosuppression, as studies have demonstrated that TSE-affected animals are immunologically responsive to other antigens following exposure to the TSE agent (Clarke, 1968; Gardiner and Marucci, 1969; Garfin et al., 1978).

Although there is no classical immune response to TSE infection, studies of naturally and experimentally scrapie-affected sheep have demonstrated elevated levels of immunoglobulin production during the clinical phase of the disease (Collis and Kimberlin, 1983; Collis et al., 1979). Furthermore, increases in serum amyloid P component in scrapie-affected mice (Coe et al., 2000), and tumour necrosis factor-α, interleukin-1β, and interleukin-6 in scrapie-affected cell lines (Bacot et al., 2003) have been detected. Recent research has also shown that murine scrapie infection is associated with the hypertrophy of FDC networks and increased numbers of mature B-lymphocytes within secondary lymphoid follicles (McGovern et al., 2004), however the ability of FDCs to trap antigen is unaltered (McBride et al., 1992). Taken together these data suggest that any alterations in the host's immune response may be related to TSE-induced immune dysfunction rather than a direct immune response to the TSE agent.

1.2.9 Chemotherapy for TSE diseases

As the conversion of PrP^{C} to PrP^{Sc} is believed by many to be the cause of TSE infection this interaction has been targeted in the development of therapeutic interventions (Aguzzi et al., 2001; Head et al., 2001; Koster et al., 2003). Many of the therapeutic interventions used are sulphated polyanions (Koster et al., 2003). PrP^{Sc} amyloid is known to contain sulphated glycosaminoglycans, it is therefore suggested that this group of drugs might competitively block the interaction between PrP and endogenous glycosaminoglycans (Caughey and Raymond, 1993). For example, Congo red (CR) is a sulfonated azo dye commonly used in histopathology to stain amyloid fibrils has been shown to inhibit the accumulation of PrP^{Sc} and infectivity in scrapie-affected mouse neuroblastoma cells (Caughey et al., 1993; Caughey and Race, 1992). In addition, administration of CR to scrapie-affected hamsters at the time of inoculation can prolong the incubation period (Ingrosso et al., 1995). However, the use of CR therapeutically is limited by its low blood-brain barrier permeability and carcinogenicity (Rudyk et al., 2000). Furthermore, recent research has now shown that CR renders PrP^{Sc} resistant to clearance by phagocytic cells *in vivo,* but has no affect on the disease incubation period in mouse models (Beringue et al., 2000a). A recent study using curcumin a non-toxic, natural compound which is structurally related to CR has found that although it is capable of inhibiting PrP^{Sc} accumulation *in vitro* it had no effect when given orally to scrapie-affected hamsters (Caughey et al., 2003). Thus, the problems associated with CR therapy are unlikely to be overcome in the immediate future.

Other drug treatments which have been shown to bind amyloid or deregulate PrP^{C} to PrP^{Sc} conversion either *in vivo* or *in vitro* include: anthracycline 4-iodo-4-deoxy-doxorubicin (IDX) (Tagliavini et al., 1997), tetrapyrroles (Brown, 1984; Caughey et al., 1998), amphotericin B (Adjou et al., 1996; Pocchiari et al., 1987), suramin (Gilch et al., 2001; Ladogana et al., 1992), tetracycline (Tagliavini et al., 2000) and β-sheet breaker peptides (Soto, 1999; Soto et al., 2000).

The anti-malarial drug quinacrine and the antipsychotic drug chlorpromazine both inhibit PrP^{Sc} formation and can cross the blood-brain barrier (Korth et al., 2001). Studies of scrapie and CJD affected rodent models have shown that a quinacrine derivative, termed quinoline, can prolong survival time (Murakami et al., 2004). However, other groups have reported no effect on survival time following quinacrine administration to murine CJD models (Collins et al., 2002). As these drugs have been previously approved for use in humans it has been possible to test their clinical application on vCJD patients. Administration of quinacrine to vCJD patients daily for 3 months resulted in a clinical improvement in patient responsiveness (Nakajima et al., 2004). A larger study, termed the Prion-1 trial, is presently underway in the UK to further investigate the use of quinacrine in human TSE diseases (http://www.ctu.mrc.ac.uk/studies/cjd.asp). Similarly, pentosan polysulphate (PS), a heparin analogue with anti-inflammatory and anti-thrombotic properties (MacGregor et al., 1984), has been shown to reduce disease susceptibility and extend survival time in rodent models over a range of times both pre- and post-inoculation (Doh-ura et al., 2004; Farquhar et al., 1999). PS has already been used in both clinical and veterinary medicine. Its use was approved by the High Court (UK) for treatment of

vCJD patients (Dyer, 2003; Mayor, 2003). Treatment with PS appeared to stabilise both patients (Dyer, 2003; Mayor, 2003). Although, a large number of potential drug candidates have been tested, currently no effective therapy exists to treat TSE diseases in animals or humans.

1.2.10 Immunisation strategies for TSE diseases

The possibility of anti-TSE immunisation strategies was first highlighted by studies which demonstrated that *in vitro* pre-incubation of anti-PrP antibodies with scrapie-affected hamster brain homogenate reduced the scrapie titre (Gabizon et al., 1988). The inhibitory effects of anti-PrP antibodies are considered to be due to the binding of the antibodies to either PrP^{C} or PrP^{Sc} molecules, blocking or modifying the conversion of PrP^{C} to PrP^{Sc} (Heppner et al., 2001b; Peretz et al., 2001; Sigurdsson et al., 2003; White et al., 2003). Anti-PrP antibodies might also block PrP^{Sc} replication by either accelerating PrP^{C} degradation (Perrier et al., 2004) or by altering PrP^{C} biogenesis (Gilch et al., 2003).

To determine whether PrP-specific antibodies were effective *in vivo*, Heppner *et al* generated mice which expressed an anti-prion protein antibody μ chain in $Prnp^{-/+}$ mice. These mice did not succumb to scrapie infection following peripheral inoculation, suggesting that the PrP-specific immunity was responsible for their protection (Heppner et al., 2001b). Others have shown that administration of anti-PrP antibodies to scrapie-affected mice can reduce levels of PrP^{Sc} and scrapie infectivity within the periphery and prolong survival time (Sigurdsson et al., 2003; White et al., 2003). Immunisation of scrapie-affected rodents with anti-PrP

antibodies either conjugated to keyhole limpet hemocyanin (Schwarz et al., 2003) or emulsified in complete Freund's adjuvant (Tal et al., 2003), both known stimuli of the immune system, have also been shown to prolong survival time. At present no immunisation strategies have been approved for use in TSE-affected animals or humans.

1.3 Transportation of TSE Agents

1.3.1 How are TSEs agents transported to the LRS?

Although extensive research has been conducted to define the role of the LRS in TSE pathogenesis following peripheral exposure, little is known of how TSE agents are transported from the site of challenge to the LRS. As FDCs are non-mobile cells situated in secondary lymphoid tissues often distant from the site of initial exposure, the TSE agent would need to be transported to FDCs either via the lymphatics to the draining lymph nodes or via the bloodstream to the spleen. A number of possible cellular candidates exist for the transportation of the TSE agent from the site of challenge to the LRS, some of which are discussed below.

1.3.2 M-cells

After oral TSE exposure immunolabelling for PrP is detected in the follicle associated epithelium (FAE) (Beekes and McBride, 2000; Heggebo et al., 2002), suggesting that the uptake of the TSE agent from the gut lumen occurs via the FAE. Membranous cells (M-cells) situated in the FAE acquire luminal material and transport it directly to the subepithelial lymphoid tissues (Neutra et al., 2001). It has been demonstrated that M-cells are able to perform transepithelial transport of

infectivity *in vitro* (Heppner et al., 2001a). Taken together these data suggest that M-cells might be an important transport mechanism for TSE agents following oral exposure.

1.3.3 Dendritic cells

Dendritic cells (DCs) are bone-marrow derived migratory cells which reside in peripheral tissues and the T-lymphocyte areas of lymphoid organs (Banchereau et al., 2000; Steinman et al., 1974). The main function of DCs is to acquire antigen with in the periphery and then transport it to the draining lymphoid tissues to be presented in a MHC class restricted manner primarily to T-lymphocytes (Banchereau et al., 2000; Banchereau and Steinman, 1998; Steinman et al., 1974). Studies have shown that the prion protein fragment $PrP_{106-126}$ is a chemoattractant for immature monocyte-derived DC (Kaneider et al., 2003), suggesting that DC might traffic to TSE affected tissues. Furthermore, studies by Huang *et al* demonstrated that DC can acquire PrP^{Sc} *in vitro* and transport intestinally administered PrP^{Sc} directly to lymphoid tissues *in vivo* (Huang et al., 2002). Thus, it is highly likely that following oral exposure DCs would be involved in the transportation of the TSE agent from the gut lumen to the gut-associated lymphoid tissues. DCs might also be likely candidates for the transportation of the TSE agent to lymphoid tissues following inoculation via other routes of peripheral exposure.

1.3.4 Macrophages

Macrophages are mobile phagocytic cells present within the majority of tissues and the blood (Roitt, 1994). Their natural function is to clear apoptotic cells and

pathogens and subsequently present antigens in a MHC class restricted manner (Roitt, 1994). However, as studies suggest that macrophages are involved in the degradation of scrapie (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982) it is unlikely this cell type is critical to the transportation of the TSE agent.

1.3.5 Lymphocytes

T-lymphocyte are unlikely candidates for transportation of TSE agents from the periphery to the LRS deficiencies in T-lymphocytes alone ($CD4^{-/-}$, $CD8^{-/-}$, $\beta 2\mu^{-/-}$, $TCR\alpha^{-/-}$ or *perforin*$^{-/-}$ mice) has no effect on disease susceptibility or the accumulation of infectivity within lymphoid tissues (Klein et al., 1997; Klein et al., 1998). Taken together these data suggest that it is unlikely that T-lymphocytes are involved in transportations of the agent.

The presence of B-lymphocytes is required for peripheral scrapie infection, as mice deficient in B-lymphocytes alone (μMT mice; (Klein et al., 1997) or jointly deficient in both mature B and T-lymphocytes (SCID, *Rag-1*$^{-/-}$, *Rag-2*$^{-/-}$ and *Arg*$^{-/-}$ mice) have impaired accumulation of scrapie infectivity within the spleen as well as impaired neuroinvasion (Brown et al., 1997c; Fraser et al., 1996; Klein et al., 1997; O'Rourke et al., 1994). Although the role of B-lymphocytes in TSE pathogenesis is likely to be due to their immunological relationship with FDCs (Chaplin and Fu, 1998; Kosco-Vilbois et al., 1997) the possibility that B-lymphocytes might be involved in the transport of TSE infectivity can not be excluded. However, no infectivity has been detected on circulating blood lymphocytes in rodent models when high levels of

infectivity are detected within lymphoid tissues (Raeber et al., 1999b), suggesting that B-lymphocytes are unlikely to be involved in the peripheral transport of the TSE agent.

1.3.6 Peripheral nerves

The initial sites in which TSE agents might enter the host (gastro-intestinal tract and the skin) (Beekes and McBride, 2000; Kimberlin and Walker, 1989a; Taylor et al., 1996a) are highly innervated tissues and therefore TSE agents might be transported directly to the CNS via peripheral nerves. Studies using transgenic mice that express PrP^C only on nervous tissue and not within their lymphoid organs have demonstrated that after oral exposure scrapie infectivity successfully reaches the CNS and induces disease (Race et al., 1995). Indeed, the relatively neurotropic TSE strain 263K is considered to directly infect nerves at the site of inoculation (Beekes and McBride, 2000; Beekes et al., 1998; McBride and Beekes, 1999; McBride et al., 2001). Furthermore, peripheral exposure to a high dose of TSE inoculum can establish TSE infection in the CNS without the requirement for replication of the agent within the LRS (Klein et al., 1997; Klein et al., 1998; Prinz et al., 2002). However, the relevance of high dose inoculum to natural TSE pathogenesis is not known. Studies have also shown that highly immunodeficient SCID mice are refractory to scrapie infection following exposure to moderate/low doses of inoculum (Fraser et al., 1996; O'Rourke et al., 1994). Thus, the role of the peripheral nerves in transport of the TSE agent may be dependent on the dose of inoculum, the strain of agent and route of exposure used.

1.3.7 TSE agent transport from the LRS to the CNS

Following accumulation and replication within the LRS it is not known how TSE agents are transported from FDCs to the PNS/CNS. The role of peripheral nerves in the transportation of the agent was first demonstrated by studies where injection of scrapie directly into the sciatic nerve by-passed the need for prior replication within the LRS and was sufficient to cause disease (Kimberlin et al., 1983a; Kimberlin et al., 1983b; Kimberlin and Walker, 1980). Since these initial experiments, several studies have demonstrated that TSE infectivity gains access to the CNS via particular neural pathways and ganglia of the peripheral nervous system (Beekes and McBride, 2000; Beekes et al., 1998; Glatzel and Aguzzi, 2000; Glatzel et al., 2001; McBride and Beekes, 1999; McBride et al., 2001). However, it is not known how scrapie infectivity reaches the PNS from FDC networks on which it accumulates. It is possible that the scrapie agent might be transported by a mobile cell type such as the DCs (Aucouturier et al., 2001). Similarly, T-lymphocytes have been proposed as a mode of transport for scrapie infectivity from the periphery to the CNS due to the detection of infiltrates of T-lymphocytes within the brains of scrapie-affected rodent models (Betmouni et al., 1996; Lewicki et al., 2003). However, this hypothesis seems unlikely as removal of the thymus or deficiencies in T-lymphocytes have no effect on disease pathogenesis (Fraser and Dickinson, 1978; Klein et al., 1997). Alternatively, recent research has demonstrated that movement of FDCs closer to peripheral nerves by disruption of chemokine gradients enhanced neuroinvasion of the scrapie agent (Prinz et al., 2003b), suggesting that transport into the PNS could occur directly from FDCs. Further research is required to determine the precise TSE agent transport mechanism between FDCs and nerves.

1.4 TSEs and the skin

1.4.1 TSE agent transmission via the skin

Studies in mice have shown that TSE exposure through scarified skin is an effective means of scrapie transmission (Taylor et al., 1996a); the skin might be a possible route of natural exposure. The potential acquisition of TSE agents via the skin highlights important health and safety issues, including whether scientists and health workers are at risk from acquiring infectivity from handling infected tissues or instruments. Previous studies have demonstrated that surgical instruments contaminated with CJD infectivity are capable of transmitting infection (Flechsig et al., 2001; Ramasamy et al., 2003) it is therefore conceivable that the skin may be the route of exposure following any surgical procedure which breaches this barrier. Furthermore, many biopharmaceutical and cosmetic products that are applied to the skin contain material from sheep and cattle sources (Birmingham, 2000; Department of Health, 2002; Lupi, 2002), which could potentially harbour TSE infectivity. Scarified skin or skin lesions might also be potential routes of exposure for scrapie in sheep (Brotherston et al., 1968) or CWD of deer (Miller et al., 2004). Understanding the immunobiology of scrapie following transmission via the skin will be important in the assessing the relative risk of exposure via this route and the development of therapeutic strategies.

1.4.2 Structure and biology of the skin

The skin covers the entire surface of the body and forms a continuous layer with the epithelial mucosa of the gastro-intestinal, respiratory and urogenital tracts. Human skin forms approximately 8 % of the total body mass and it thickness can range from

1.5 – 4 mm depending on its location and maturation (Champion et al., 1991). The skin provides a continuous physical barrier that protects the internal systems from mechanical, chemical, thermal, photic, osmotic and microbial damage. The skin is responsible for many diverse functions including: biochemical synthesis of Vitamin D, production of cytokines and growth factors, primary immunosurveillance and sensory surveillance (Millington and Wilkinson, 1983).

The skin is comprised of two main layers, the epidermis and dermis, although some texts may refer to a third layer termed the subcutaneous layer composed mainly of fat (Fig.1.4). The epidermis is divided into five strata from deep to superficial; stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and the stratum corneum (Williams, 1995). The majority of the epidermis is comprised of keratinocytes. Keratinocytes are terminally differentiated from mitotically active basal cells within the stratum basale to non-viable cornified cells of the stratum corneum (Kligman, 1964). As these cells migrate upwards from the stratum basale to the corneum (approximately over a 28 day period) there is a progressive increase in the synthesis of keratin proteins. Melanocytes are also present within the epidermis, which are interspersed among keratinocytes within the stratum basale. Melanosomes (pigment containing granules produced by melanocytes) are transferred to surrounding keratinocytes providing them with protection from ultra-violet light as they progress upwards through the strata (Sagebiel and Odland, 1972). Merkel cells are also present within the stratum basale in association with nerve endings (Winkelmann, 1973). These neuroendocrine cells function as mechanoreceptors. Finally, a sub-population of dendritic cells termed Langerhans

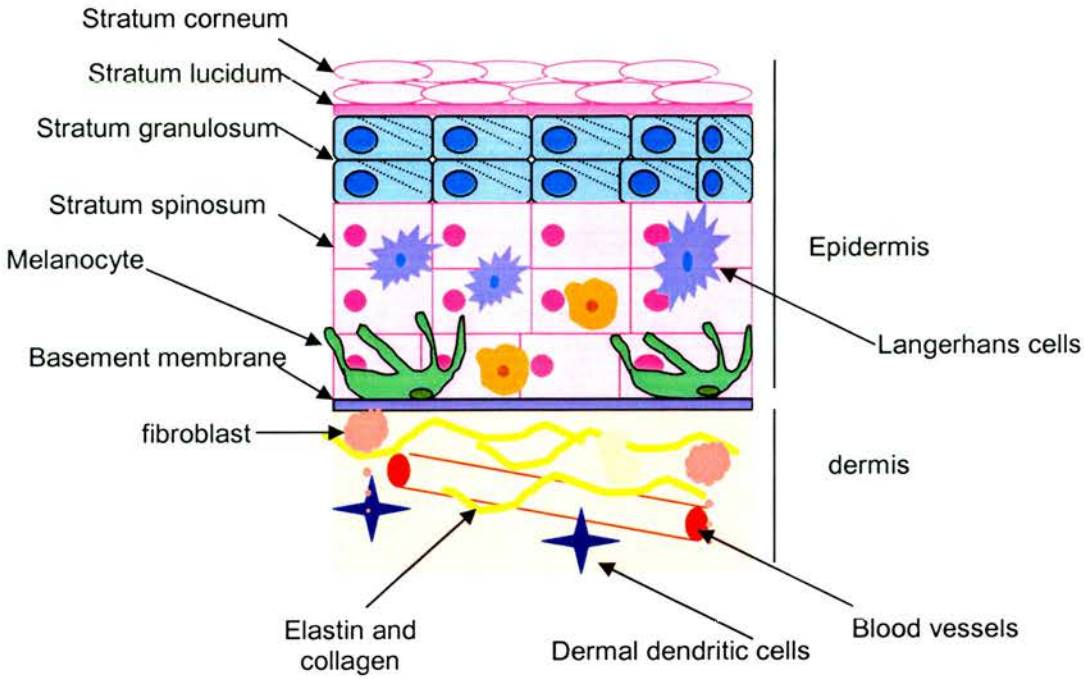


Figure 1.3- A schematic representation of the layers of the skin.

cells (LCs) are present within the basal and spinosum strata (Langerhans, 1868). Langerhans cells are responsible for immunosurvelliance and immunological responses within the skin. Occasionally a very small number of lymphocytes are found within the deepest layers of the epidermis but they are not considered a resident cell population.

The dermal layer of the skin is separated from the epidermal layers by a basement membrane comprised of the membranes of basal cells, collagen and lamina filaments. The dermis consists of two layers, the papillary dermis and the reticular dermis, although transition between the two is gradual (Williams, 1995). Both layers consist of dense networks of collagen fibres and elastin fibres and proteoglycans (Hay, 1981) interwoven by blood vessels and nerve fibres. The dermis contains two major categories of cells: fixed cells and free cells. Fixed cells include, fibroblasts, endothelial cells, nerve cells and muscle cells. Whereas, free cells whose percentages can vary according to anatomical location and immune status include, macrophages, dermal dendritic cells, eosinophils, neutrophils, mast cells and B and T-lymphocytes. The skin also can contains pilosebaceous units which contain the hair shaft and its follicle and its associated arrector pili muscle and sebaceous gland which are all located within the dermis although the hair shaft extends out through the epidermis.

1.4.3 Antigen transport from the skin

After a pathogen has entered the skin there are several mechanisms by which it may be transported out with this microenvironment. Pathogen invasion of the skin leads

to the release of pro-inflammatory cytokines such as TNF-α and interleukins (IL) by keratinocytes and LCs within the epidermis and mast cells, dendritic cells and macrophages within the dermis (Kupper and Fuhlbrigge, 2004). The subsequent sequence of events and relative proportion of cell types involved can vary depending on the type of antigen present. In general, LCs and dermal DCs acquire antigens present at the site of exposure and are stimulated to mature by the cytokine and chemokine signals released by surrounding cells. They then emigrate from the skin to the draining lymph node where they present antigen in a MHC-class restricted manner to naïve T-lymphocytes to initiate a primary immune response (Lappin et al., 1996). The release of pro-inflammatory cytokines and chemokines by cells within the skin also attracts macrophages, NK cells and neutrophils from the dermal post-capillary venule to the site of injury. The main function of neutrophils and macrophages is the phagocytosis and enzymatic digestion of pathogenic bacteria. Macrophages also possess the ability to present antigen in a MHC-class restricted manner to memory T-lymphocytes either at the site of injury or by migrating to the draining lymphoid tissues. Antigens are also transported from the skin in a cell free-dependent manner as oposonised components of the complement cascade (Pepys, 1976). Opsonised antigen is transported within the tissue fluids of the skin and subsequently passes into the lymphatic vessels and drains to the nearest draining lymph node. Antigens can also leave the skin via the blood vessels ultimately with the potential to invade distant tissues from the site of exposure as is observed following exposure to malaria (Pouniotis et al., 2004). The skin is also highly innervated, and some pathogens such as rabies virus (Coulon et al., 1989) and the

herpes simplex virus (Worrell and Cockerell, 1997) have been shown to directly infect peripheral nerves.

1.4.4 Candidate mechanisms for the transport of the scrapie agent from the skin

The majority of cells that reside in the skin are non-mobile, in terms of movement outwith the skin and therefore unlikely to be directly involved in the transport of scrapie infectivity. However, both LCs and macrophages are highly mobile cells and both possess the ability to acquire antigen (Ag) in the periphery and transport it to the nearest draining lymph nodes (DLNs). It is therefore conceivable that either of these cell types could transport scrapie infectivity from the skin to lymphoid tissues. Both *in vivo* and *in vitro* studies have demonstrated the capacity of macrophages to reduce scrapie infectivity rather than aid its transmission (Beringue et al., 2000b; Carp and Callahan, 1982; Jeffrey et al., 2000b), suggesting these cells would be an unlikely transport mechanism from the skin.

The genetic or temporary depletion of complement components, prior to peripheral inoculation of rodent models, with the scrapie agent, results in a significantly prolonged incubation period (Klein et al., 2001; Mabbott et al., 2001). Furthermore, these studies demonstrate that complement components are involved in the localization of scrapie infectivity to FDCs (Klein et al., 2001; Mabbott et al., 2001), suggesting that the transport of scrapie infectivity from the skin to the LRS might be cell free. In addition, peripheral nerves could directly take up the scrapie agent however, this seems unlikely, as SCID mice are refractory to scrapie infection following inoculation via the skin (Taylor et al., 1996).

The most likely candidate for the transportation of the scrapie agent from the skin to the LRS are LCs. Research by Huang *et al* demonstrated that migrating intestinal DCs can acquire and transport PrP^{Sc} from the gut lumen through the lymphatics to the LRS (Huang et al., 2002). As LCs are immature dendritic cells, it is conceivable that LCs might perform the same function following exposure to the scrapie agent via the skin. In addition, studies using the neurotoxic prion protein fragment $PrP_{106-126}$ have shown that this peptide is a chemoattractant for immature DCs (Kaneider et al., 2003) and can also induce up-regulation of pro-inflammatory cytokines such as IL-1β and TNFα following exposure (Bacot et al., 2003). Taken together these data suggest that LCs might have the potential to transport the scrapie agent from the skin to the LRS.

1.4.5 Langerhans cells

LCs (Langerhans, 1868) are a population of immature DCs located above the basal layer of epithelial cells in the skin, oral, nasal, esophageal, pulmonary, vaginal and rectal mucosa. Within the skin, LCs constitute approximately 2-4% of all epithelial cells and usually reside within the stratified spinosum epithelia (Prickle cell layer) of the epidermis. LCs originate from bone-marrow progenitors (Elbe et al., 1989) and circulate in the peripheral blood before residing within the skin (Strunk et al., 1997). LCs can be identified within the epidermis according to their dendritic morphology and the presence of the intracytoplasmic organell, termed the Birbeck granule (Birbeck et al., 1961), although not all LCs have this structure (Mommaas et al., 1994). LCs can also be identified based on their surface phenotype, as they are the

only cell population within the normal epidermis that expresses MHC class II (Klareskog et al., 1977 794).

LCs are the sentinels of the skin, consistently sampling their microenvironment for both self and non-self Ag. Upon Ag-encounter immature LCs are induced to undergo phenotypic and functional changes that culminate in the complete transition from Ag-capturing cells to a mobile Ag-presenting cell (APC). During this maturation, LCs leave their cutaneous compartment and migrate to the draining lymph node where they present antigen in a MHC-class restricted manner to naïve T-lymphocytes initiating primary immune responses (Lappin et al., 1996).

1.4.6 Antigen acquisition and processing by LCs

LCs express a wide range of surface molecules that allow them to recognize and acquire Ag (Banchereau and Steinman, 1998). For example, Toll-like receptors (TLRs) 2 and TLR-4 have been recently shown to be expressed by LCs and are able to directly bind LPS, whereas TLR-9 expressed by LCs binds unmethylated CpG motifs (Mitsui et al., 2004). Simiarly, C-type lectins such as Langerin, Dectin-2 and CD205 bind pathogen associated sugar residues via highly conserved carbohydrate recognition domains (Figdor et al., 2002). Studies have also shown that LCs also express complement receptors (C1qR and CR3; (Burke and Gigli, 1980)) and Fc-receptors (FcγRII/III; (Esposito-Farese et al., 1995)) both of which facilitate the uptake of Ag-coated in immune complexes. In addition, LCs are also able to bind apoptotic cells via integrin components (Albert et al., 1998; Berman et al., 2003). Once captured, Ag can be internalized via several different mechanisms such as

phagocytosis (Reis e Sousa et al., 1993), macropinocytosis (Sallusto and Lanzavecchia, 1995) and receptor mediated endocytosis (Jiang, 1995). The Ag then enters the endocytic pathway of the cell and is directed towards late-endosomal compartments for loading onto MHC molecules (Banchereau and Steinman, 1998). Furthermore, previous studies have shown that DCs are capable of secreting proteases to process extracellular Ag which is then directly loaded onto MHC molecules at the cell surface (Santambrogio et al., 1999). However, not all Ags are degraded by DCs as studies have shown that LCs are able to acquire and retain unprocessed Ag and transfer them to naïve B-lymphocytes to initiate an immune response (Wykes et al., 1998).

1.4.7 LC activation and migration

The production of various cytokines by both LCs and keratinocytes plays a key role in the induction of LC activation and migration to the DLN (Luger and Schwarz, 1990). In response to certain stimuli, such as application of contact allergens (Silberberg-Sinakin et al., 1976), low-dose UVB radiation (Bergstresser et al., 1980), invasion of pathogens or necrotic cells (Banchereau and Steinman, 1998) both cell types produce a variety of cytokines such as IL-1α, IL-1β, IL-18 GM-CSF, TNF-α and macrophage inflammatory proteins (MIPs) (Lappin et al., 1996). The cytokines produced first following stimulation and considered the most critical to LC migration are TNF-α and IL-1β (Cumberbatch et al., 2001; Cumberbatch et al., 1997b; Cumberbatch et al., 1999; Cumberbatch and Kimber, 1992). IL-1β, which is primarily a product of LC, is delivered in an autocrin fashion via IL-1 receptor (Cumberbatch et al., 1997a; Cumberbatch et al., 1997b; Cumberbatch et al., 1999).

In contrast, TNF-α is derived from the surrounding keratinocytes and signals through the TNF-R2 on the LC (Cumberbatch and Kimber, 1992; Wang et al., 1996; Wang et al., 1997). In the absence of either of these two cytokines active LC migration is impaired (Cumberbatch et al., 1997b).

LCs are attached to keratinocytes via E-cadherin (Jakob and Udey, 1997). Following the production of the fore mentioned cytokines a decline in the expression of mRNA for E-cadherin occurs allowing LCs to dissociate (Jakob and Udey, 1997). Furthermore, LCs up-regulate adhesion molecules such as intergrin proteins (Price et al., 1997) and ICAM-1 (Cumberbatch et al., 1992), which guide LCs to the basement membrane of the dermis. The secretion of metalloproteinases by LCs allows partial digestion of the basement membrane to facilitate their passage to the afferent lymphatics (Kobayashi, 1997).

Upon activation and migration LCs express the chemokine receptor CCR7 (Saeki et al., 1999) which mediates their migration through the afferent lymphatics towards the DLN through the coordinated actions of several chemokines such as 6Ckine and macrophage inflammatory protein-3β (Kellermann et al., 1999). During this migration, LCs develop a mature phenotype becoming potent APCs. Upon arrival at the DLN mature APCs present Ag in a MHC-restricted manner to T-lymphocytes and stimulate their proliferation and differention via the co-stimulatory molecules CD40, CD80 and CD86 (Banchereau et al., 2000). The production of IL-12 by APCs polarizes the differentiation of T-lymphocytes into cytotoxic Th1 cells, which are involved in cell-mediated immunity (Cella, 1996; Koch, 1996; Reis e Sousa,

1997). Alternatively, IL-4 production by APCs polarizes T-lymphocytes to proliferate and differentiate into Th2 cells, which aid humoral immunity (Hayashi et al., 2000).

1.4.8 LC and pathogen interactions

Following transmission via the skin, several pathogens are known to exploit LCs either to gain transport to the lymphoid tissues or to avoid immune detection (Rescigno and Borrow, 2001). Human immunodeficiency virus has been shown to use CCR5 receptor expressed by LCs to infect these cells and then utilizes their migration pathway to facilitate transport to T-lymphocyte regions of lymphoid tissues where it preferential infects $CD4^+$ T-lymphocytes (Kavanagh and Bhardwaj, 2002; Kawamura et al., 2003; Reece et al., 1998). Similarly, LCs have been shown to be involved in the transport of cytomegalovirus (CMV) to lymphoid tissues (Hertel et al., 2003). This virus also down-regulates MHC class II expression by LCs by interfering with the transcription and trafficking of MHC (Hertel et al., 2003), presumably to hinder the priming of virus specific T-lymphocytes. Other pathogens which utilize LCs include, dengue virus (Wu et al., 2000), maedi-visna lentivirus (Ryan et al., 2000), Venezuelan equine encephalitis virus (MacDonald and Johnston, 2000) and Leishmania major (von Stebut et al., 1998), demonstrating the variety of pathogens which can exploit LC functions.

1.5 Aims

Studies by Taylor and colleagues have shown that skin scarification is an effective means of scrapie agent transmission in mice (Taylor et al., 1996a). Understanding

the immunobiology of the scrapie agent transmission via the skin will help to determine the likelihood of transmission of natural TSEs via this route and aid the development of therapeutic strategies. A functional immune system is critical for the transmission of scrapie to the CNS following skin scarification, as SCID mice are refractory to scrapie infection, (Taylor et al., 1996a). These data demonstrate that infectivity is unable to reach the CNS directly via the blood or peripheral nerves. Instead, it is likely that infectivity accumulates and replicates in the LRS prior to neuroinvasion, as observed with other peripheral routes of exposure (Hilton et al., 1998; Kimberlin and Walker, 1989a; McBride et al., 1992; Sigurdson et al., 1999; van Keulen et al., 1996).

With this in mind the overall aim of this study was to utilize a mouse-passaged scrapie model to investigate the immunobiology of TSEs following transmission via the skin. Specifically this study addresses the following issues:

1) To determine whether the scrapie agent accumulates in the LRS following transmission via the skin and if so, what cells are critical for the accumulation of the agent in the LRS.
2) To determine how the scrapie agent is transported from the skin to the LRS

Aim 1:

Studies using SCID mice have suggested that there is a requirement for the LRS after transmission of the scrapie agent via the skin (Taylor et al., 1996a). However, it is not known whether the scrapie agent accumulates within lymphoid tissues prior to neuroinvasion. To address this issue, tissue samples were collected at various time

points after inoculation and infectivity levels measured (Chapter 3). Furthermore, it is not known which cells within the LRS are critical for the accumulation of the scrapie agent following transmission via the skin. The most likely candidate cells involved in replication and accumulation of infectivity in the LRS are FDCs, which are critical for the efficient transmission of the disease to the CNS following other peripheral routes of inoculation (Brown et al., 1999; Mabbott et al., 2000b). In order to address this issue two separate approaches were taken. Firstly, a chimeric mouse model was used that had a mismatch in PrP status between FDCs and other bone-marrow derived cells within the LRS (Brown et al., 1999) to determine the separate roles FDCs and lymphocytes (Chapter 4). Secondly, FDCs were temporarily dedifferentiated either prior to, or shortly after, inoculation to determine the role of FDCs in neuroinvasion (Chapter 5).

Aim 2:

Previous research has suggested that DCs have the potential to transport the scrapie agent (Huang et al., 2000). It is therefore conceivable that LCs, a sub-population of DCs situated in the epidermis, could transport the agent from the skin to the LRS after inoculation via the skin. LCs were exposed to scrapie-affected brain homogenate *in vitro* to determine whether LCs have the potential to acquire PrP^{Sc} and scrapie infectivity (Chapter 6). Secondly, mouse models were utilised which had impaired LC migration to the DLN. Using these models the role of LCs in the transportation of the scrapie agent from the skin to the DLN was investigated (Chapter 7).

2

Materials and Methods

2.1 Mouse strains

All mice used in these studies were housed under specific pathogen free conditions in a conventional animal facility with a 12 hrs light and 12 hrs dark cycle. Mice were used between 8-12 weeks of age and individual experiments were age-matched. All experiments were conducted under the provisions of the Animals (Scientific Procedures) Act 1986.

2.1.1 C57BL/Dk

This mouse strain has been extensively used at the Neuropathogenesis Unit (NPU), Institute for Animal Health, Edinburgh in scrapie transmission studies and as indicator mice in incubation period bioassays to determine scrapie infectivity titres.

2.1.2 129/Ola mice and *Prnp*$^{+/+}$ mice

The 129/Ola mouse strain was used at the NPU as a background strain for the generation of *Prnp*$^{-/-}$ mice. The Prnp deficient mouse model (*Prnp*$^{-/-}$ mice) was generated on the 129/Ola mouse background by insertion of a neomycin resistance cassette into exon 3 of the *Prnp* gene (Manson et al., 1994a).

2.1.3 SCID/*Prnp*$^{+/+}$ mice

The SCID/*Prnp*$^{+/+}$ mouse line was originally produced as described by Brown *et al* (Brown et al., 1999), by crossing Severe Combined Immunodeficient mice (SCID) (C.B-17 *scid/scid*, MHC type H-2^{d}) with *Prnp* deficient mice (129/Ola *Prnp*$^{-/-}$ mice, MHC type H-2^{b}) (Manson et al., 1994a). At the F2 generation, mice were selected for the SCID phenotype, MHC type H-2^{b} and either the *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ genotype.

2.1.4 CD40 ligand deficient mice

CD40 ligand (*Tnfsf5*) deficient ($CD40L^{-/-}$) mice (Xu et al., 1994) were a kind gift from Prof. David Gray, University of Edinburgh, UK. These mice were generated by the insertion of a neomycin resistance cassette into exons 3 and 4 of the *Tnfsf5* gene. Mice were backcrossed and maintained on a C57BL/6 background. Age-matched C57BL/6 mice were used as wild-type controls for experiments using these immunodeficient mice.

2.2 Mouse genotype and phenotype confirmation

2.2.1 Preparation of chromosomal DNA

Portions of tail tissue (approximately 10 mm long) or spleen tissue fragments (approximately 5 mg) were incubated in 800 µl of lysis buffer (1.5 M sodium acetate, 1 M Tris-HCl, pH 8, 0.5 M EDTA, 10% SDS, 7 µg/ml Proteinase K: (Roche Diagnostics, Lewis, UK)) and gently shaken for 16-18 hrs at 37°C. Following incubation, 600 µl of phenol: chloroform: isoamyl alcohol (25:24:1, Sigma, Poole, UK) was added to the lysate and vortexed thoroughly. The samples were then centrifuged at 13,000 x *g* for 5 mins and supernatant fractions collected. To the supernatant, 20 µl of 3 M sodium acetate and 600 µl of isopropanol were added. The samples were then mixed by inversion several times before being left to stand for 10 min at room temperature. Samples were then centrifuged at 13,000 x *g* for 1 min and the supernatant discarded. The resulting DNA pellet was washed in 70% ethanol/water (vol/vol) and resuspended in 100 µl of sterile DNase and RNase free water. DNA preparations were stored at 4°C prior to analysis.

2.2.2 Determination of *Prnp* genotype by the polymerase chain reaction

The *Prnp* genotypes of bone-marrow grafted animals were determined by PCR analysis, through the amplification of the *Prnp* gene and a portion of the neomycin resistance gene, to detect the presence of the $Prnp^{-/-}$ genotype. The PCR mixture, (total volume 60.7 µl) contained; 5 µl 10X PCR buffer, 5.0 µl 50mM $MgCl_2$, 1.0 µl 10mM dNTP mix (Life Technologies, Paisley, UK), 1 µl chromosomal DNA, 0.5 µl (100 pmol/µl) each of primers 1, 2, 3 and 4, 0.2 µl Taq polymerase (5U/µl) (Life Technologies) and 50 µl sterile DNase and RNase free water. The sequences of the oligonucleotide primers for *Prnp* gene were as follows:

Primer 1. *Prnp* Fwd- 5'- ATG GCG AAC CTT GGC TAC TGG CTG- 3'

Primer 2. *Prnp* Rev- 5'- TCA TCC CAG GAT CAG CAA GAT GAG- 3'

Sequence positions are taken from Entrez entry U29186 (Lee et al., 1998). Primer 1 anneals to position 28690-28713 and primer 2 to 29454-29431. The above forward and reverse primers anneal to the start and stop codons of the *Prnp* gene open reading frame, respectively. These primer pairs were designed to generate a fragment of 750 bp.

The sequences of the oligonucleotide primers for the mouse neomycin resistance gene were as follows:

Primer 3. *Neo* Fwd- 5'- TTG AGC CTG GCG AAC AGT TC - 3'

Primer 4. *Neo* Rev- 5' – GAT GGA TTG CAC GCA GGT TC - 3'

Sequence positions are taken from Entrez entry M77786 (unpublished). Primer 3 anneals to position 4860-4878 and primer 4 to 5371-5352. The above primers anneal to the neomycin resistance gene within $Prnp^{-/-}$ mice, which is located within exon 3

of the *Prnp* gene (Manson et al., 1994a). These primer pairs were designed to generate a fragment of 550 bp.

Following a hot start at 94°C for 3 mins an amplification cycle was carried out for 30 cycles at the following temperatures; 94°C 50 sec, 62°C 50 sec, 72°C 50 sec on a thermal cycler (Genius PCR System, Techne, Cambridgeshire, UK). A final extension period at 72°C for 10 mins was included at the end of the 30 cycles. PCR products were resolved electrophoresis at 125V through a 1.5% agarose gel containing 1 μg/ml ethidium bromide.

2.2.3 Determination of *Tnfsf5* genotype by the polymerase chain reaction

The *Tnfsf5* genotype of C57BL/6 (wild-type) and $CD40L^{-/-}$ deficient mice was determined by PCR analysis, through the amplification of a portion of the *Tnfsf5* gene or a portion of the neomycin resistance gene inserted into exon 3 and 4 of the *Tnfsf5* gene. The PCR mixture, (total volume 60.7 μl) contained; 5 μl 10X PCR buffer, 1.5 μl 50mM $MgCl_2$, 1.0 μl 10mM dNTP mix (Life Technologies), 1 μl chromosomal DNA, 0.5 μl (100 pmol/μl) each of the primers 1, 2, 3 and 4, 0.2 μl Taq polymerase (5U/μl) (Life Technologies) and 50 μl sterile DNase and RNase free water. The sequences of the oligonucleotide primers for the*Tnfsf5* gene were as follows;

Primer 1. *Tnfsf5* Fwd-5'- CCC AAG TGT ATG AGC ATG TGT GT-3'

Primer 2. *Tnfsf5* Rev-5'- GTT CCT CCA CCT AGT CAT TCA TC-3'

Sequence positions are taken from Entrez entry AL672128.8 (unpublished). Primer 1 anneals to position 146478-146500 and primer 2 anneals to position 146740-

146718. The above forward and reverse primers anneal to introns 4 and 5 of the*Tnfsf5* gene respectively. These primer pairs were designed to generate a of fragment 250 bp.

The sequences of the oligonucleotide primers for the mouse neomycin resistance gene were:

Primer 3. Neo Fwd-5'- GCC CTG AAT GAA CTG CAG GAC-3'

Primer 4. Neo Rev-5'- GGG TAG CCA ACG CTA TGT C-3'

Sequence positions are taken from Entrez entry M77786. Primer 3 anneals to position 5218-5197 and primer 4 to 4708-4726. The above forward and reverse primers anneal to exons 3 and 4 of the neomycin resistance gene respectively. These primer pairs were designed to generate a of fragment 500 bp.

Following a hot start at 94°C for 1.5 mins an amplification cycle was carried out for 12 cycles at the following temperatures; 94°C 20 sec, 64°C 30 secs, 72°C 35 secs, followed by a second amplification cycle carried out for 25 cycles at the following temperatures; 94°C 20 sec, 58°C 30 secs, 72°C 35 secs. A final extension period at 72°C for 10 mins was included at the end of the second amplification cycle. All PCR products were resolved by electrophoresis at 125 V through a 1.5% agarose gel containing 1 μg/ml ethidium bromide.

2.2.4 Enzyme-linked immunoabsorbent assay (ELISA) for mouse immunoglobulins (Ig)

For analysis of Ig content, sera was diluted (1/1000) in carbonate bicarbonate buffer (0.5 M, pH 5) and absorbed to flat bottomed, medium binding microwell plates

(Costar®, High Wycombe, UK) for 18-24 hrs at 4°C. Plates were then blocked with 0.01 M PBS (pH 7.5) containing 1% bovine serum albumin (BSA). Bound Ig was detected using horseradish peroxidase conjugated rabbit anti-mouse Ig (DAKO, Cambridgeshire, UK). Bound peroxidase activity was measured by incubation with o-phenylenediamine as a substrate (Sigma). Optical density (OD) was measured at 490 nm using a V-max kinetic microplate reader (Molecular devices, CA, USA). A standard curve was generated by serial dilutions of polyclonal mouse Ig (1mg/ml) (Serotec, Oxford, UK) and was used to convert the OD values of experimental samples to mg of Ig/ml of sera. Sera from wild-type immunocompetent mice and SCID mice were used as reference controls.

2.3 Pre- inoculation manipulations/treatments

2.3.1 Bone-marrow grafting of SCID/*Prnp*$^{+/+}$ mice

Bone-marrow from the femurs and tibias of adult 129/Ola mice or *Prnp*$^{-/-}$ mice (section 2.1.2) (Manson et al., 1994a) was prepared as single-cell suspensions ($3x10^7$- $4x10^7$ viable cells/ml) in Hank's balanced salt solution (HBSS) (Life Technologies). Recipient SCID/*Prnp*$^{+/+}$ mice (section 2.1.3) were reconstituted with 0.1 ml bone-marrow by injection into the tail vein.

2.3.2 Caspase-1 inhibitor treatment

Where indicated, before inoculation with the scrapie agent by skin scarification groups of C57BL/Dk mice were pre-treated at the shaved inoculation site with 50 µl of 0.4 mM caspase-1 inhibitor II (Ac-YVAD-cmk; Calbiochem, Beeston, UK), 0.4 mM caspase-3 inhibitor III (Ac-DEVD-cmk; Calbiochem) or vehicle alone Dimethyl

sulfoxide (DMSO) using a previously established protocol (Antonopoulos et al., 2001). One hour later mice were inoculated with the scrapie agent as described in section 2.4.2. Care was taken to ensure that the scrapie agent was only applied to the pre-treated area of skin.

2.3.3 LTβR-Ig treatment

Where indicated, either 3 days before, or 14 or 42 days after inoculation with the scrapie agent by skin scarification, groups of C57BL/Dk mice were given a single 100 μg intra-peritoneal (i.p.) injection of a fusion protein containing the soluble lymphotoxin β receptor (LTβR) domain linked to the Fc portion of human IgG1 (LTβR-Ig) (Force et al., 1995) or 100 μg of polyclonal human IgG (hu-Ig; Sandoglobulin®). Both reagents were a kind gift from Jeff Browning (Biogene Inc, Cambridge, MA, USA).

2.4 Inoculation with the scrapie agent

2.4.1 Scrapie strains

The ME7 scrapie strain was used for all inoculations conducted in this thesis. ME7 was originally isolated from the spleen of a Suffolk sheep affected with natural scrapie (Zlotnik and Rennie, 1963). The strain was originally passaged into mice by intragastric challenge of Moredun random bred mice. The agent was then passaged intracerebrally (i.c.) into Moredun mice followed by 9 passages in inbred C57BL/Dk mice at NPU.

The mouse-passaged ME7 strain of scrapie has been well characterised and produces prominent neuronal vacuolation. This scrapie strain has been used extensively at NPU for the study of peripheral scrapie pathogenesis in mice. After peripheral inoculation with moderate or low dose of ME7 (1.0% or 0.1% brain homogenate) there is an obligate requirement for amplification of the agent in lymphoid tissues prior to neuroinvasion (Brown et al., 1999; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978; Fraser and Farquhar, 1987; Mabbott et al., 2000a; Mabbott et al., 2002; Mabbott et al., 2000b; Mabbott et al., 2003; Taylor et al., 1996a) This scrapie strain is therefore the most appropriate strain to use to investigate peripheral pathogenesis of scrapie infection after inoculation via the skin.

2.4.2 Inoculation with the scrapie agent

Mice were inoculated with the ME7 scrapie strain by skin scarification of the medial surface of the right thigh. Prior to scarification approximately 1 cm^2 area of hair covering the site of scarification was trimmed using curved scissors and then removed completely with an electric razor. Twenty-four hours later a 23-gauge needle was then used to create a 1 cm long abrasion of the upper epidermal layers of the skin at scarification site. Then using a 26-gauge needle one droplet (~6 µl) of ME7 scrapie inoculum from either a 1% or 0.1% (wt/vol) dilute of terminally scrapie-affected mouse brain homogenate in physiological saline, was applied to the abrasion and worked into the site using sweeping strokes. The scarification site was then sealed with OpSite (Smith & Nephew Medical Limited, Hull, UK) and allowed to dry before returning the animals to their final holding cages. Where indicated some mice were inoculated intra-cerebrally (i.c.) with 20 µl of a 1 % scrapie-affected

mouse brain homogenate in physiological saline (a dose of approximately 1 x $10^{4.5}$ intracerebral units, 50 % infectious dose {ID_{50} units}) as a titre control. Following inoculation, animals were coded and assessed weekly for signs of clinical disease and killed at a standard clinical end-point (as described in section 2.4.3) (Fraser and Dickinson, 1973). Scrapie diagnosis was confirmed by histopathological assessment of vacuolation in the brain (section 2.4.4).

2.4.3 Determination of scrapie incubation period

The scrapie incubation period is defined as the time elapsed (in days) between the day of inoculation and the clinical endpoint of the disease (Dickinson et al., 1968; Fraser and Dickinson, 1973). At NPU all mice are clinically monitored by experienced animal technicians and scoring of clinical symptoms begins approximately 120 days after inoculation dependent on the route of exposure. Inoculated mice are assessed weekly on the same day and each mouse is given a rating as follows; "unaffected", "possibly affected" or "definitely affected". The clinical endpoint is defined in one of four ways: 1) the day on which the mouse receives a second consecutive "definite" rating; 2) the day on which a mouse receives a third "definite" rating within four consecutive weeks; 3) the day on which the mouse is culled in extremis or 4) the mouse is found dead in its cage after receiving a "definite" rating at the time of scoring the previous week. This system has been used for many years at NPU and has been applied to a wide range of TSE experiments.

2.4.4 Lesion profiles

Whole brains were cut into five defined areas (Fraser and Dickinson, 1968), fixed in 2% paraformaldehyde-periodate-lysine (0.1 M periodate; 0.075 M D-L Lysine; 2% paraformaldehyde in 0.05 M phosphate buffer pH 7.4) and embedded in paraffin wax. Sections (thickness, 6 μm) were stained with hematoxylin and eosin. Vacuolation in the brain was scored on a scale of 0-5, by experienced histology staff, in the following grey-matter (G1-G9) and white-matter (W1-W3) areas (Fraser and Dickinson, 1973): G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decoction of superior cerebellar peduncles; W3, cerebral peduncles.

2.4.5 Bioassay of scrapie infectivity in tissues samples

At various time points after challenge mice were sacrificed and tissues taken to determine the scrapie infectivity titre. For bioassay of scrapie infectivity in the spleen, half spleens were pooled from 2-4 mice from each group and prepared as 10 % (wt/vol) homogenates in physiological saline and 20 μl injected i.c. into groups of up to 12 C57BL/Dk indicator mice. Similarly, inguinal lymph nodes draining the site of challenge were pooled from 2-4 mice from each group and prepared as 10% (wt/vol) homogenates. The scrapie titre in each sample was determined from the mean incubation period response curves for scrapie-infected spleen tissue (Dickinson et al., 1969). To compare the levels of scrapie infectivity remaining at the site of skin scarification, skin (approximately 1 cm^2) from the inoculation site was

homogenized in 200 µl of physiological saline in a Ribolyser (Hybaid, Middlesex, UK). Samples were pooled from 4 mice and 20 µl injected i.c into groups of up to 12 C57BL/Dk indicator mice.

2.5 *Ex vivo* analysis of tissues

2.5.1 Immunohistochemical analysis of lymphoid tissues

To monitor follicular dendritic cell (FDC) status, spleen or inguinal lymph nodes were snap-frozen at the temperature of liquid nitrogen. Serial frozen sections (thickness, 10 µm) were cut on a cryostat and FDCs visualized by staining with one or more of the following antibodies: FDC-specific rat monoclonal antibodies FDC-M1 (BD Pharmingen, Oxford, UK), FDC-M2 (AMS Biotechnology, Oxon, UK), 8C12 monoclonal antiserum to detect complement receptor 1 (CD35) (BD PharMingen).

B-lymphocytes were detected using either rat monoclonal antibody RA3-6B2 specific for CD45R (Caltag, Towcester, UK) or the lectin peanut agglutinin (PNA), which preferentially binds to galactosyl (β 1-3) N-acetylgalactosamine residues (t-Antigen) on blasting germinal centre B-lymphocyte glycoproteins (Rose, 1980) (Vector Laboratories, Burlingame, CA, USA).

For the detection of PrP in spleen tissue or inguinal lymph nodes, tissues were fixed in 2% paraformaldehyde-periodate-lysine (0.1 M periodate; 0.075 M D-L Lysine; 2% paraformaldehyde in 0.05 M phosphate buffer pH 7.4) and embedded in paraffin wax. Sections (thickness, 6 µm) were deparaffinized, and pre-treated to enhance PrP

immunostaining by immersion in water (55°C for 30 mins), and subsequent immersion in formic acid (98%) for 5 min (Tricia McBride personal communication). Sections were then stained with the PrP-specific rabbit polyclonal antiserum 1B3 (Appendix 1) (Farquhar et al., 1989)

All immunolabeling for the above antibodies was carried out using alkaline phosphatase coupled to the avidin-biotin complex (Vector Laboratories). Vector Red (Vector Laboratories) was used as a substrate. All sections were counterstained with hematoxylin to distinguish cell nuclei.

2.5.2 Immunohistochemical analysis of brain tissue

For the detection of PrP in brain tissue, tissues were fixed in periodate-lysine-paraformaldehyde (0.1 M periodate; 0.075 M D-L Lysine; 2% paraformaldehyde in 0.05 M phosphate buffer pH 7.4) and embedded in paraffin wax. Sections (thickness, 6 μm) were deparaffinized, and pre-treated to enhance PrP immunostaining by hydrated autoclaving (15 min, 121°C), and subsequent immersion in formic acid (98%) for 5 min (Kitamoto et al., 1991). Sections were then stained with the PrP-specific mouse monoclonal antibody 6H4 (Prionics, Zurich, Switerland) (Appendix 1) and immunolabelling was carried out using the DAKO EnVision™$^{+}$ System (DAKO,). Diaminobenzidine (DAB) (Sigma) was used as a substrate.

Glial fibrillary acid protein (GFAP) was detected on adjacent brain sections using rabbit GFAP-specific antibody (DAKO) and immunolabelling carried out using

alkaline phosphatase coupled to the avidin-biotin complex (Vector Laboratories). Vector Red was used as a substrate (Vector Laboratories). All sections were counterstained with hematoxylin to distinguish cell nuclei.

Microglia were detected by staining with biotin-conjugated *Lycopersicon esculentum*-agglutinin (LEA; Sigma), which preferentially binds to poly-N-acetyl lactosamine residues on microglial cells (Acarin et al., 1994). Immunolabelling was carried out using hydrogen peroxidase coupled to the avidin-biotin complex with DAB as a substrate.

2.5.3 Paraffin embedded tissue (PET) immunoblots.

For detection of PrP^{Sc} in spleen and inguinal lymph nodes, tissues were fixed in 2% paraformaldehyde-periodate-lysine and embedded in paraffin wax. Serial sections (thickness 6μm) were mounted on polyvinylidine difluoride membranes (PVDF, 0.45μm-pore; Bio-Rad, Hemel, Hempstead, UK) and fixed by incubation at 55°C overnight. PET blotting was carried out as previously described (Schulz-Schaeffer et al., 2000). Briefly PVDF membranes were deparaffinised and digested with 20 μg/ml of proteinase K (10 mM Tris-HCl pH 7.8, 100 mM NaCl and 0.1% Brij) for 16 hrs at 55°C. Membranes were then washed in TBS/Tween (10 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.5% Tween) before being denatured in 3 M guanidine isothiocyanate (10 mM Tris-HCl pH 7.8) for 10 mins. Prior to immunodetection membranes were blocked in 2% casein. PrP was detected with the PrP specific rabbit polyclonal antiserum 1B3 (Farquhar et al., 1989) (Appendix 1) in blocking buffer for 2 hrs, followed by alkaline phosphatase-conjugated goat anti-rabbit

Immunoresearch Laboratories Inc, West Grove, PA, USA), and bound alkaline phosphatase activity detected with SigmaFast™ NBT/BCIP solution (Sigma). PET blots were assessed using an Olympus dissecting microscope.

2.5.4 Immunoblot detection of PrP^{Sc}

Individual spleen fragments or pooled inguinal lymph nodes (two pooled from each mouse assayed) were prepared as previously described by Farquhar (Farquhar et al., 1994). Briefly, before immunoblot analysis, tissue homogenates were treated with 80 μg proteinase K (to confirm the presence of PrP^{Sc}) and subsequently partially purified by treatment with 2% (wt/vol) N-lauroylsarcosine (in 0.1 M Tris pH 7.4) allowing sedimentation only of the proteinase-K-resistant, detergent-insoluble fraction of PrP (PrP^{Sc}). Samples were subjected to electrophoresis through sodium dodecyl sulphate 10-20% polyacrylamide gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad) by semi-dry blotting. PrP was detected with the PrP-specific mouse monoclonal antibody 8H4 (Zanusso et al., 1998) (Appendix 1). Immunolabelling was carried out using horseradish peroxidase conjugated rat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc), and bound horseradish peroxidase activity detected with Supersignal® West Dura Extended Duration Substrate (Pierce, Chester, UK).

Where indicated PrP was also detected in earlier experiments (Chapter 4; Fig. 4.9) with the PrP-specific rabbit polyclonal antiserum 1B3 (Farquhar et al., 1989)(Appendix 1) followed by alkaline phosphatase-conjugated goat anti-rabbit

antibody (Jackson ImmunoResearch Laboratories Inc), and bound alkaline phosphatase activity detected with SigmaFast^{TM} NBT/BCIP solution (Sigma).

2.6 Cell lines

2.6.1 Culture of cell lines

The XS106, XS52 and NS47 cell lines used in this research were a kind gift from Akira Takashima (Dept. Dermatology, University of Texas, U.S.A). The XS52 and XS106 cell lines are long term dendritic cell lines established from the epidermis of newborn mice (Timares et al., 1998; Xu et al., 1995a). The XS52 and XS106 cell lines were cultured in complete RPMI 1640 medium (cRPMI) (Gibco BRL, Paisley, UK) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 0.25 μg/ml antibiotic-antimycotic solution, 50 μM 2-β-mercaptoethanol, 1% non-essential amino acids (Sigma) and 10% heat-inactivated fetal calf serum (Invitrogen, Paisley, UK). NS47 cells belong to the fibroblast lineage and are cultured in cRPMI as described above (Schuhmachers et al., 1995). Culture media for the XS52 cell lines was further supplemented with 2 ng/ml mouse recombinant granocyte macrophage colony-stimulating factor (GM-CSF: R&D Systems, Abingdon, UK) and 10% (v/v) culture supernatant derived from the NS47 cell line. The culture media used to culture the XS106 cells was supplemented with 0.5 ng/ml mouse recombinant GM-CSF (R&D Systems) and 5% culture supernatant derived from the NS47 cell line. All cell lines were cultured at 37°C in 5% CO_2/air atmosphere.

2.6.2 Passage and storage of cell lines

Upon receipt of the cell lines, cells were cultured and passaged 4 times to obtain a large batch culture. Cells were then removed from the culture vessels using cell dissociation solution (1% EDTA; Sigma). Cells were then washed in cRPMI and resuspended at $2x10^5$ cells/ml of freezing media {10% dimethyl sulphoxide (Sigma) and 90% heat-activated fetal calf serum (Invitrogen)}and stored in a Nalgene cryogenic freezing container (Nalgene, Hertford, UK) at -80°C for 24 hrs. Aliquots were then stored in liquid nitrogen until required. For each experiment a new vial of cells was revived and seeded at the density appropriate for the culture vessel being used (Table 2.1). Cells were grown for 10 days in culture prior to use in experiments. All cells were grown and passaged in the same manner to ensure homogeneity between cell populations in individual experiments.

Table 2.1 Seeding density of cell lines

Cell Culture Vessel	Volume of Media (mls)	Number of Cells
24 well plate	1 ml per well	$5x10^5$
25 cm^2 flasks	5 mls	$6x10^5$
75 cm^2 flasks (NS47 cells)	10 mls	$3x10^5$

2.6.3 Cell viability and metabolic activity assay

Aliquots of cells were diluted 1/10 in trypan blue solution (Sigma). Viable cells were identified by the exclusion of trypan blue and viable cell numbers determined using a heamocytometer. The metabolic activity of cell lines was also determined by

the colorimetric AlamarBlue™ assay (Serotec) as per manufactures instructions. The AlamarBlue system contains an oxidation-reduction (REDOX) indicator that changes colour in response to the chemical reduction of growth medium resulting from cellular metabolic activity. Colour change was measured by absorbance at 570 nm and 600 nm using a V-max kinetic microplate reader (Molecular devices).

2.7 Characterisation of cell lines

2.7.1 Flow cytometry analysis

Cells were harvested and viable cell numbers determined by trypan blue exclusion (section 2.6.3). Cells were centrifuged at 1,400 rpm 4°C for 10 mins and resulting pellets resuspended in FACS buffer (PBS pH 7.4 containing 0.1% BSA, 0.1% sodium azide and 0.02% EDTA) to give $1x10^6$ cells per 100 µl of buffer. Cells were blocked for 15 mins at room-temperature with 0.1µg of mouse monoclonal antibody CT-17.2 (Caltag) which specifically reacts with CD16.2/CD32.2 allotypic variants of the mouse FcγIII/II receptor. Cells were then incubated with one of the following FITC-conjugated monoclonal antibodies specific for either: CD40 (clone 3/23), CD80 (clone RMMP-1), CD86 (clone RMMP-2), MHC II-A^k (clone 14V.18), CD205 (clone NLDC-145) (all from Serotec), CD11c (clone HL3), CD54 (clone 3E2) (BD Biosciences) or 8H4 (PrP- Appendix 1; (Zanusso et al., 1998)) for 1 hour at 4°C. Immunolabeling of 8H4 was carried out using goat anti-mouse FITC-conjugated antibody for 45 mins at 4°C (Caltag). The appropriate FITC-conjugated antibodies (Serotec) were used as non-specific Ig-isotype controls. All antibodies were titrated prior to use to determine their saturation point. All samples were fixed in 1% paraformaldehyde and flow cytometry conducted on a FACSCalibur™

(Becton Dickinson, San Jose, CA, USA) and data analysed using CELLQuest™ (Becton Dickinson)

.

2.7.2 Reverse transcription polymerase chain reaction analysis of *Prnp* mRNA expression

mRNA was isolated from cell lines using μMACS mRNA Isolation Kit (Miltenyi Biotec Ltd, Bergisch Gladbach, Germany) as per the manufacturer's instructions. cDNA was synthesised from 100 ng of mRNA. Briefly, 1μl oligo $(dT)_{12-18}$ primer (25 μg: Life Technologies) and 12 μl DEPC-treated water was added to the mRNA and incubated at 70°C for 10 mins. 4 μl 5X First strand buffer, 2 μl DTT (0.1M) and 1μl dNTP (10mM) (Life Technologies) was then added and incubated at 42°C for 2 mins. Finally 1 μl of Superscript II (Life Technologies) was added and the mixture incubated at 42°C for for 50 mins. The reaction was stopped by incubation at 70°C for 15 mins. The expression of *Prnp* was determined through PCR amplification of the cDNA. The PCR mixture, (total volume 60.2 μl) contained; 5 μl 10X PCR buffer, 1.5 μl 50 mM MgCl, 1 μl 10 mM dNTP mix (Life Technologies), 1 μl cDNA, 0.5 μl (100pmol/μl) each of specific primers, 0.2 μl Taq polymerase (Life Technologies) and 50 μl sterile DNase and RNase free H_20. The sequences of the specific primers used were as follows;

Primer combination 1.

1.*Prnp* Fwd-5'- AGG TTA ACT GAA CCA TTT CAA CCG AGC-3':

2 *Prnp* Rev-5'- TCC CCC AGC CTA GAC CAC GAG A-3'

Primer combination 2

1*Prnp* Fwd-5'- AGG TTA ACT GAA CCA TTT CAA CCG AGC-3':

3 *Prnp* Rev-5'- GCT TGT TCC ACT GAT TAT GG-3'

Sequence positions are taken from Entrez entry U29186 (Lee et al., 1998). Primer 1 anneals to position 10869-10890 in exon 2. Primer 2 anneals to position 29506-29485 in the 3' untranslated region and primer 3 anneals to position 28990-28977 in exon 3. Primers 1 and 2 are designed to generate a fragment of 1000 bp and primers 1 and 3 to generate a fragment of 400 bp.

Following a hot start at 94 °C for 3 mins an amplification cycle was carried out for 30 cycles at the following temperatures; 94 °C 30 sec, 63 °C 30 sec, 72 °C 60 sec on a thermal cycler. A final extension period at 72 °C for 10 mins was included at the end of the 30 cycles. PCR products were resolved by gel electrophoresis at 125V through a 1.5% agarose gel containing 1 μg/ml ethidium bromide.

2.7.3 Reverse transcription polymerase chain reaction analysis of TLR-2 and TLR-4 mRNA expression

mRNA was isolated from XS106 cells using the μMACS mRNA Isolation Kit (Miltenyi Biotec, Germany) as per the manufacturer's instructions. cDNA was synthesised from 100 ng of mRNA. Briefly, 1μl oligo $(dT)_{12-18}$ primer (25 μg: Life Technologies) and 12 μl DEPC-treated water was added to the mRNA and incubated at 70°C for 10 mins. 4 μl 5X First strand buffer, 2 μl DTT (0.1M) and 1μl dNTP (10mM) (Life Technologies) was then added and incubated at 42°C for 2 mins. Finally 1 μl of Superscript II (Life Technologies) was added and the mixture incubated at 42°C for 50 mins. The reaction was stopped by incubation at 70°C for

15 mins. The expression of TLR-2 and TLR-4 was determined through PCR amplification of the cDNA. The PCR mixture, (total volume 60.2 µl) contained; 5 µl 10X PCR buffer, 1.5 µl 50 mM MgCl, 1 µl 10 mM dNTP mix (Life Technologies), 1 µl cDNA, 0.5 µl (100 pmol/µl) each of specific primers, 0.2 µl Taq polymerase (Life Technologies) and 50 µl sterile DNase and RNase free H_20. The sequences of the specific primers used were as follows;

***TLR-2* Fwd**-5'- GTCTCTGCGACCTAGAAGTGGA-3'

***TLR-2* Rev**-5'- CGGAGGGAATAGAGGTGAAAGA-3'

***TL- 4* Fwd**-5'- GCAATGTCTCTGGCAGGTGTA-3'

***TLR-4*-Rev**-5'- CAAGGGATAAGAACGCTGAGA-3'

Sequence positions for TLR-2 are taken from Entrez entry AF165189 (Lin et al., 2000). The forward primer anneals to position 126-147 and the reverse primer anneals to position 162-140. Sequence positions for TLR-4 are taken from Entrez entry NM_021297 (Dissanayake et al., 2004). The forward primer anneals to position 949-968 and the reverse primer anneals to position 1354-1334. Primers for TLR-2 are designed to generate a fragment of 336 bp and primers fro TLR-4 are designed to generate a fragment of 406 bp.

Following a hot start at 94 °C for 2.5 mins an amplification cycle was carried out for 32 cycles at the following temperatures; 94 °C 30 sec, 56 °C 30 sec, 72 °C 45 sec for TLR-2. The optimum cycling conditions for TLR-4 were 95 °C 45 sec, 61 °C 45 sec, 72 °C for 45 sec, for 36 cycles. All reactions were carried out on a thermal cycler and a final extension period at 72 °C for 10 mins was included at the end of

the cycles. PCR products were resolved by gel electrophoresis at 125V through a 1.5% agarose gel containing 1 μg/ml ethidium bromide.

2.8 Analysis of cell lines after exposure to the scrapie agent

2.8.1 Preparation of brain homogenates for *in vivo* experiments

Brains used as inocula in cell culture experiments were removed aseptically from terminally scrapie-affected C57BL/Dk mice. Age-matched, non-infected C57BL/Dk mice were used as negative controls. Four whole brains were pooled from each group and homogenised in a sterilised Griffith homogeniser in cRPMI (section 2.6.1) to give a 10% (wt/vol) homogenate. Aliquots were stored at -80°C until required.

2.8.2 Exposure of cells to the scrapie agent and lipopolysaccaride

Sterile glass coverslips were placed into the wells of 24 well plate (Corning, Bucks, UK) and cells were plated at $1x10^5$ cells per well. When cultures were confluent 10 μl of 10% brain homogenate (approximately 1 mg of brain homogenate) was added to each well. Cells were then incubated for 16 hrs at 37°C in 5% CO_2/ air atmosphere. Residual homogenate was then removed by washing the cells with cRPMI by pipetting and fresh media added. Cells were then maintained for, 24, 48, 72 or 96 hrs in culture with no further fresh media added. Where indicated cells were also stimulated with 10 ng/ml of lipopolysaccharide (LPS serotype 055:B5: Sigma) one hour before exposure to the scrapie agent. Cells were maintained in culture and treated in the same manner as stated above.

2.8.3 Immunofluorescent confocal microscopy

XS106 cells were exposed to the scrapie agent as described in section 2.8.2 for the times indicated. Cells were then washed and cytospin preparations prepared at a cell density of approximately $1x10^5$ per slide. Cytospins were fixed in methanol at -20°C for 20 mins and non-specific binding sites blocked with 3% BSA for 1 hour. Further blocking of non-specific binding was achieved by addition 0.1μg of mouse monoclonal antibody CT-17.2 (FcγII/III; Caltag). Avidin/biotin binding sites were subsequently blocked with the avidin/biotin blocking kit (Vector Laboratories) as per manufacture's instructions. PrP was detected using the PrP-specific mouse monoclonal antibody 8H4 (Zanusso et al., 1998) (Appendix 1), followed by biotin conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Immunolabelling was carried out using Strepavidin Alexa 488 (Biosciences, Cambridge, UK). Cell nuclei were distinguished using Dapi nucleic acid stain (Biosciences).

2.8.4 Cell immunoblot detection of PrP^{Sc}

PVDF membranes (Biorad) were treated with 100% methanol and then soaked in lysis buffer (0.5% deoxycholate, 0.5% Triton X-100, 150 mM NaCl, 150 mM Tris HCl pH 7.5). After exposure to the scrapie agent (section 2.8.2) for the allocated time period coverslips were removed and placed cell-side down on PVDF membranes (Biorad). Filter paper, which had been previously soaked in lysis buffer, was used as a cassette for the transfer and a glass plate used to apply pressure. The coverslips were removed and the membranes left to air dry for 1 hr. Blots were stored at –20 °C prior to analysis.

For detection of PrP, membranes were rehydrated in lysis buffer and treated with proteinase-K (10 μg/ml in 0.1 M Tris-HCl pH 7.4) and then denatured with 3 M guanidine isothiocyanate. To confirm the presence of PrP^{Sc} on membranes as previously described (Bosque and Prusiner, 2000). After the above pre-treatments membranes were probed with either PrP-specific mouse monoclonal antibody 8H4 or 7A12 (Zanusso et al., 1998) (Appendix 1), followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Bound horseradish peroxidase activity was detected with Supersignal® West Dura Extended Duration Substrate (Pierce). After exposure, membranes were stained with 0.5 μg/ml ethidium bromide and observed in UV light to confirm transfer of the cell layer

2.8.5 Determination of scrapie infectivity in cell lysates

Cells were dislodged by a rubber policeman and cell suspensions collected. Viable cell numbers were determined by trypan blue exclusion. Cells were washed twice in HBSS. Cell pellets were resuspended in appropriate volume of sterile saline to give $2x10^5$ cells per 20 μl dose. Lysis of cells was achieved by 10 successive rapid freeze-thaw cycles at the temperature of liquid nitrogen. Non-infected cells or cells exposed to normal brain homogenate were used as reference controls. Samples were stored at -80°C until use. Cell lysates were injected i.c. (20 μl) into groups of up to 12 C57BL/Dk indicator mice. The scrapie titre in each tissue was estimated from the mean incubation period response curves for scrapie-infected spleen tissue (Dickinson et al., 1969).

2.9 Statistical Analysis

All data is presented as the mean ± S.E.M. The statistical significance of differences in means between experimental groups was calculated using ANOVA one-way analysis with Minitab software. A p-value ≤ 0.05 was considered to be significantly different.

Infectivity titres as determined by incubation period assay in indicator mice have a standard deviation of ± 0.5 log i.c. ID_{50}/g, this margin of error was used as a threshold above or below which differences between samples were considered significant.

3

The time course and targeting of scrapie infectivity following transmission via the skin

3.1 Abstract

After peripheral exposure, TSE infectivity and PrP^{Sc} usually accumulate in lymphoid tissues prior to neuroinvasion. Studies in mice have shown that scrapie exposure through scarified skin is an effective means of scrapie transmission. Following inoculation via the skin a functional immune system is critical for the transmission of the scrapie agent to the brain, as severe combined immunodeficiency mice are refractory to infection. Until now, it has not been known whether scrapie infectivity accumulated in the skin and the lymphoid tissues after inoculation via the skin. To investigate this, mice were inoculated with the scrapie agent by skin scarification and skin and lymphoid tissues were collected at various times after inoculation. Infectivity titres in pooled tissues homogenates were then measured by incubation period assay in groups of indicator mice. Data presented here demonstrates that scrapie infectivity does not replicate in the skin following skin scarification but instead accumulates and replicates in the draining lymph node (DLN). Trace levels of infectivity were detected in the DLN after inoculation, which then steadily increased from 7 days post-exposure, reaching plateau levels at approximately 42 days after inoculation. At 42 days after inoculation, infectivity had spread to the non-draining lymph node and the spleen, most likely via the blood.

3.2 Introduction

The involvement of the lymphoreticular tissues (LRT) in scrapie pathogenesis was first demonstrated after subcutaneous inoculation of mice with the Chandler scrapie strain (Eklund et al., 1967). Assays of various tissues at sequential time points after inoculation with scrapie-affected brain homogenate demonstrated that infectivity was first detectable in the spleen and peripheral lymph nodes 28 days after inoculation, and accumulated to high levels within these tissues prior to detection within the spinal cord or the brain (Eklund et al., 1967). Similarly, following intra-peritoneal or oral transmission of the ME7 scrapie strain, infectivity accumulates in LRT before spreading to the central nervous system (CNS) (Brown et al., 1999; Fraser, 1996; Mabbott et al., 2000b). The accumulation of the scrapie agent in LRT is critical for disease pathogenesis as survival time is significantly extended if the spleen is removed before intra-peritoneal inoculation (Fraser and Dickinson, 1970; Fraser and Dickinson, 1978).

The natural acquisition route of scrapie is unknown, however, studies of naturally scrapie infected sheep have shown that PrP^{Sc} is first detected in the Peyer's patches and gut-associated lymphoid tissues, prior to its detection within other lymphoid tissues and the CNS (Andreoletti et al., 2000; Heggebo et al., 2002; Heggebo et al., 2000; van Keulen et al., 1996). Thus, demonstrating the involvement of the LRT in TSE pathogenesis in species other than rodents Subsequent studies, have demonstrated the involvement of the LRT of vCJD patients (Hilton et al., 1998), chronic wasting disease of deer and elk (Sigurdson et al., 1999) and transmissible mink encephalopathy of mink (TME) (Hadlow et al., 1987). However, the LRT

appears not to be involved in all TSE disease as BSE in cattle (Somerville et al., 1997a) and sporadic CJD in humans (Hill et al., 1999) appear to be confined to the nervous tissues. However PrP^{Sc} is detectable in the LRT of sCJD patients at the end stage of the disease (Glatzel et al., 2003). Furthermore, PrP^{Sc} is detectable in the LRT of rodents and sheep experimentally infected with BSE (Farquhar et al., 1996; Foster et al., 2001). Thus, the involvement of the LRT appears to be strain and species dependent (Farquhar et al., 1996; Foster et al., 2001; Hill et al., 1999; Somerville et al., 1997a).

Studies in mice have shown that exposure to the scrapie agent through scarified skin is an effective means of transmission (Taylor et al., 1996a). A functional immune system is critical for the transmission of the scrapie agent from scarified skin to the brain as severe combined immunodeficiency mice are refractory to infection via this route (Taylor et al., 1996a). However, in their studies Taylor *et al* did not investigate whether scrapie infectivity accumulated within the LRT after inoculation via the skin (Taylor et al., 1996a). Determining the sequence of events following skin scarification is important to the understanding of the immunobiology of scrapie infection and will aid in the subsequent development of therapeutic strategies.

With these points in mind, experiments were undertaken to determine whether scrapie infectivity accumulates in the LRT following inoculation via the skin and the timing of targeting of infectivity to these tissues.

3.3 Results

3.3.1 Inoculation of mice via skin scarification and tissue collection.

To determine whether scrapie infectivity accumulated within the skin and the LRT following inoculation via skin scarification, C57BL/Dk mice were each inoculated with a 1% dilution of brain homogenate from a terminally scrapie-affected mouse by skin scarification of the right thigh (Chapter 2; section 2.4.2). At each of the times indicated after inoculation, skin from the site of inoculation, draining right inguinal lymph nodes (ILNs), non-draining left ILNs and the spleen were collected from 4 mice. As a control, a group of 12 mice was left to develop clinical scrapie. All these mice succumbed to disease with a mean incubation period of 308 ± 4 days. Infectivity titres in pooled tissue (n = 4) homogenates were determined by incubation period assay by intra-cerebral injection into groups of up to nine C57BL/Dk indicator mice (Chapter 2; section 2.4.5). Previous studies have suggested that the dose-response curves used to estimate infectivity titres should be specific for the tissue being assayed (Robinson et al., 1990). However, in this study infectivity titres were estimated using a spleen dose-response curve (Chapter 2; section 2.4.5), as dose-response curves were not available for ME7 scrapie infected skin and ILNs.

3.3.2 Scrapie infectivity in the skin at the site of challenge

As skin tissue had not been previously used in incubation period bioassay experiments a homogenate of pooled sterile skin (n = 4) spiked with 24 µl of terminally scrapie-affected brain homogenate was included as a control. All assay mice inoculated with spike skin homogenate developed clinical signs of scrapie at approximately 198 ± 6 days post-inoculation (dpi), representing an approximate

scrapie infectivity titre of 6.5 log i.c. 50% infectious dose (ID_{50})/g (Table 3.1; Fig 3.1). In contrast, only low levels of infectivity were detected in the skin at 30 mins after inoculation (Table 3.1; Fig 3.1). The majority of assay mice developed clinical scrapie at approximately 251 ± 3 dpi, suggesting an infectivity titre of approximately 4.0 log i.c. ID_{50}/g (Table 3.1; Fig 3.1). Similarly, at 24 hrs after inoculation all assay mice developed clinical scrapie at approximately 238 ± 6 dpi, suggesting an infectivity titre of approximately 4.5 log i.c. ID_{50}/g (Table 3.1; Fig 3.1. At 7 days after inoculation only trace levels of infectivity were detected in the skin, as only 2/6 assay mice developed clinical signs of scrapie at 257 ± 5 dpi, suggesting an infectivity titre of approximately ≤ 3.9 log i.c. ID50/g (Table 3.1; Fig 3.1).

3.3.3 Accumulation of scrapie infectivity within the draining inguinal lymph node

Scrapie infectivity was undetectable in the right ILN 30 mins after inoculation (Table 3.2; Fig 3.1). All assay mice remained free from the signs of clinical scrapie infection up to 282 dpi, suggesting an infectivity titre if present, below ≤ 3.7 log i.c. ID_{50}/g (Table 3.2; Fig 3.1). However, by 24 hrs after inoculation trace levels of infectivity were detected as 1/5 assay mice developed clinical disease (Table 3.2; Fig 3.1). At 7 days after inoculation the infectivity titre had risen to approximately 4.2 log i.c. ID_{50}/g (Table 3.2; Fig 3.1). By 21 days after inoculation the level of infectivity in the right ILN had increased to 5.5 log i.c. ID_{50}/g, which was significantly greater than the level measured at 7 days after inoculation as determined by statistical analysis of disease incubation periods for each group of indicator mice ($P = 0.001$). By 42 days after inoculation the level of infectivity in the right ILN had

Table 3.1- Scrapie infectivity in pooled samples (*n* = 4) of skin tissue taken from the site of inoculation.

Time post-inoculation	Incidence[a]	Mean incubation period (days) ± S.E.M	Titre[b]
Spike[c]	6/6	198 ± 6	6.5
30 mins	7/7	251 ± 3,	4.0
24 hrs	8/8	238 ± 6,	4.5
7 days	2/6	257 ± 5, 4X > 308	≤3.9

[a;] Incidence = number of animals affected/number of animals tested. The notation "N X > 308" means the mice were free of the signs of scrapie up to at least this time after inoculation.

[b;] Scrapie infectivity titres expressed as log i.c. 50% infectious dose units/g tissue.

[c;] 24 µl of terminally scrapie-affected brain homogenate was added to a pooled (*n* = 4) thigh skin homogenate from un-inoculated mice.

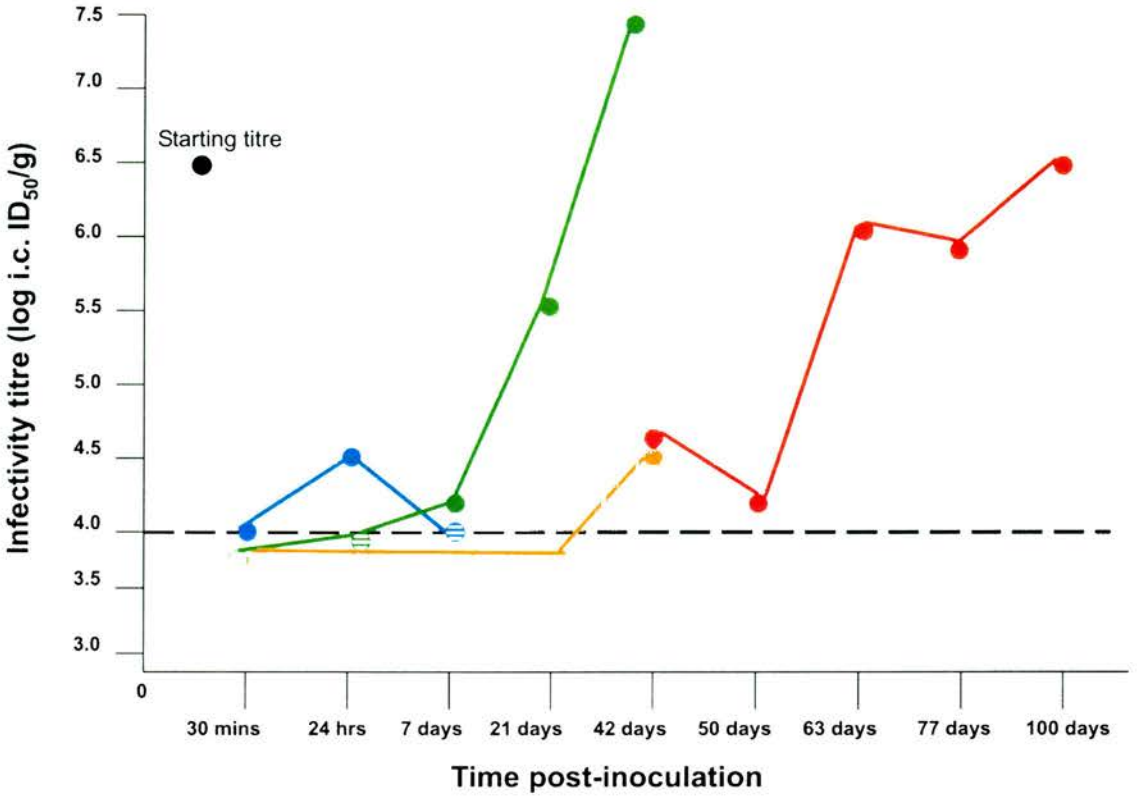


Figure 3.1- Scrapie infectivity titres in pooled samples (n = 4) of skin (blue) at the site of challenge, draining right inguinal lymph nodes (green), left non-draining inguinal lymph nodes (yellow) and spleens (red) at various times after inoculation with the scrapie agent via skin scarification. Closed circles represent groups of assay mice in which the majority of assay mice succumb to scrapie infection. Hatched circles represent groups of assay mice where approximately half of the mice succumb to scrapie infection and open circles represent groups of assay mice in which less than half or no mice succumb to scrapie infection. • starting titre of the original terminally scrapie-affected brain homogenate used to inoculate mice via the skin. Scrapie titres expressed as log i.c 50% infectious dose units/ gram tissue

Table 3.2- Scrapie infectivity in pooled samples (n = 4) of right inguinal lymph nodes draining the site of inoculation.

Time post-inoculation	Incidence[a]	Mean incubation period (days) ± S.E.M	Titre[b]
30 mins	0/7	7X > 308	≤ 3.7
24 hrs	1/5	251, 4X >308	≤ 3.9
7 days	7/8	255 ± 3, 1X > 308	4.2
21 days	6/6	218 + 5	5.5
42 days	9/9	197 ± 2	7.4

[a;] Incidence = number of animals affected/number of animals tested. The notation "N X > 308" means the mice were free of the signs of scrapie up to at least this time after inoculation.

[b;] Scrapie infectivity titres expressed as log i.c. 50% infectious dose units/g tissue.

increased a further 100 fold to 7.4 log i.c. ID_{50}/g (Table 3.2; Fig 3.1), which was significantly higher than the level measured at 21 days after inoculation ($P = 0.001$).

3.3.4 Accumulation of scrapie infectivity within the non draining inguinal lymph node

In contrast to the right ILN, trace levels of scrapie infectivity were detectable within the left ILN 30 mins after inoculation however, only 1/9 assay mice developed clinical scrapie (Table 3.3; Fig 3.1). At 24 hrs, 7 days and 21 days after inoculation all assay mice remained free from the signs of the disease up to 282 dpi, suggesting a scrapie infectivity titre if present below ≤ 3.7 log i.c. ID_{50}/g (Table 3.3; Fig 31.). By 42 days after inoculation, detectable levels of infectivity were present all assay mice, which developed clinical disease at approximately 248 ± 7 dpi, suggesting an infectivity titre of 4.5 log i.c. ID_{50}/g (Table 3.3; Fig 3.1).

3.3.5 Accumulation of scrapie infectivity within the spleen

Spleens were analysed from 42-100 days after inoculation. Experiments demonstrated that low levels of scrapie infectivity (approximately 4.1- 4.6 log i.c. ID_{50}/g) were detectable in the spleen between days 42-50 after inoculation (Table 3.4; Fig 3.1). Significantly higher levels of infectivity were detected by 63 days after inoculation (6.0 log i.c. ID_{50}/g; $P = 0.001$). Furthermore, the levels of infectivity in the spleen had reached plateau levels by 63 days after inoculation (Table 3.4; Fig 3.1), as no significant difference in infectivity levels in the spleens taken from mice between 77-100 days after inoculation was observed (range 5.9 -6.5 log i.c. ID_{50}/g)

when compared to levels in spleens taken at 63 days after inoculation ($P = 0.870$ and $P = 0.206$ respectively; Table 3.4; Fig 3.1).

Table 3.3- Scrapie infectivity in pooled samples (n = 4) of non-draining left inguinal lymph nodes at serial time points.

Time post-inoculation	Incidence[a]	Mean incubation period (days) ± S.E.M	Titre[b]
30 mins	1/9	271, 8X > 308	≤ 3.8
24 hrs	0/9	9X >308	≤ 3.7
7 days	0/9	9X > 308	≤ 3.7
21 days	0/6	6X > 308	≤ 3.7
42 days	9/9	248 ± 7,	4.5

[a]; Incidence = number of animals affected/number of animals tested. The notation "N X > 308" means the mice were free of the signs of scrapie up to at least this time after inoculation.

[b]; Scrapie infectivity titres expressed as log i.c. 50% infectious dose units/g tissue.

Table 3.4- Scrapie infectivity in pooled spleen samples (*n* = 4).

Time post-inoculation	Incidence[a]	Mean incubation period (days) ± S.E.M	Titre[b]
42 days	7/8	235 ± 3, 1X > 308	4.6
50 days	8/8	246 ± 7	4.1
63 days	8/8	208 ± 5	6.0
77 days	7/7	210 ± 8	5.9
100 days	7/9	198 ± 5, 2X > 308	6.5

[a]; Incidence = number of animals affected/number of animals tested. The notation "N X > 308" means the mice were free of the signs of scrapie up to at least this time after inoculation.

[b]; Scrapie infectivity titres expressed as log i.c. 50% infectious dose units/g tissue.

3.4 Discussion

To determine the time course and targeting of scrapie infectivity following inoculation via the skin, skin at the site of inoculation, right draining ILNs, left non draining ILNs and spleens were collected at various times after inoculation and infectivity titres estimated by incubation period assays in indicator mice. Data presented in this chapter demonstrates that only trace levels of scrapie infectivity were present in the skin between 24 hrs - 7 days after inoculation. However, scrapie infectivity did first accumulate in the draining ILN where it accumulates to high levels prior to targeting the non-draining ILN and the spleen at approximately 42 days after inoculation.

Previous studies have shown that both keratinocytes and Langerhans cells which reside within the epidermal layers of the skin express the host protein PrP^C (Pammer and Tschachler, 2002; Pammer et al., 1998; Sugaya et al., 2002). Thus, these cell types might be the first cellular targets for TSE replication following exposure via the skin. It was important to establish whether scrapie infectivity replicated within the skin after inoculation, as this could have possible implications with regard to the spread of infectivity iatrogenically, as many medical procedure breach the skin barrier. Furthermore, shedding of infected skin by animals could lead to possible environmental contamination and subsequent spread of infection. Data presented in this chapter demonstrates that low levels of scrapie infectivity are detectable within the skin at 30 mins and 24 hrs after inoculation, and only trace levels of infectivity are detectable in the skin at 7 days after inoculation. This demonstrates that scrapie infectivity does not replicate in the skin following inoculation via skin scarification.

These data are in agreement with the hypothesis that PrP^C expression alone is not the only cellular requirement for the replication of the scrapie agent (Montrasio et al., 2001; Raeber et al., 1999a). It is possible that the decline in scrapie infectivity within the skin over the 7 day observation period will be due to shedding of infectivity from the skin in association with dead skin cells. However, data presented here suggests it is also probable that some infectivity is transported from the skin to the draining ILN as the decline in scrapie infectivity within the skin corresponds with the subsequent increase in scrapie infectivity within the draining ILN.

Antigens are normally transported out of the epidermis to the draining lymph node within the first 24 hours of exposure (Kimber et al., 1999; Lappin et al., 1996; Silberberg-Sinakin et al., 1976). Following intra-ocular challenge with scrapie strain ME7, infectivity is detectable within the draining lymph nodes 24 hrs after inoculation (Fraser, 1996). In contrast, in this study only trace levels of scrapie infectivity were detectable within the draining ILN at 24 hrs after inoculation and did not increase to measurable levels until 7 days after inoculation. However, following peripheral challenge the time course of accumulation of scrapie infectivity within the draining lymph node varies with route challenge and strain of agent administered (Eklund et al., 1967; Farquhar et al., 1994; Fraser, 1996; Kimberlin and Walker, 1989b). For example: following either intra-peritoneal or subcutaneous challenge with either the 139A or Chandler scrapie strains experiments have shown that scrapie infectivity is not detectable within the draining lymph nodes until 21 days after inoculations (Eklund et al., 1967; Kimberlin and Walker, 1989b). Thus, following inoculation via skin scarification accumulation of detectable levels of infectivity

within the draining ILN is slower than that observed following intra-ocular inoculation (Fraser, 1996) but faster than that observed following either intra-peritoneal(Kimberlin and Walker, 1989a) or subcutaneous challenge (Eklund et al., 1967)

Previous studies have traditionally pooled both the right and left draining lymph nodes for determination of scrapie infectivity titres. Therefore, in these studies it was not possible to determine which lymph node was targeted first following inoculation (Eklund et al., 1967; Farquhar et al., 1994; Fraser, 1996; Kimberlin and Walker, 1989b). In this study, right and left lymph nodes were pooled separately to allow the targeting of scrapie infectivity to individual lymph nodes after inoculation to be determined. Data presented here demonstrate that scrapie infectivity firsts targets the draining lymph node prior to targeting the non-draining lymph node following inoculation by skin scarification. Furthermore, similar levels of scrapie infectivity are detected within the left ILN and spleen at 42 days after inoculation. As the left ILN and the spleen are anatomically distinct this suggests that infectivity might be spread heamogenously from the draining ILN. Taken together, these data show that the draining ILN has a significant contribution to the initial accumulation of scrapie infectivity following transmission via the skin. Comparable targeting has been reported in goats and mink following subcutaneous challenge (Hadlow et al., 1974; Hadlow et al., 1987). Following subcutaneous inoculation of goats with the scrapie agent infectivity is first detected primarily within the lymph node draining the site of inoculation (Hadlow et al., 1974). Following challenge of mink with the TME agent the peripheral replication of infectivity is confined to the lymph node draining the

site of challenge and infectivity is only detectable within other lymphoid tissues after infection has spread to the CNS (Hadlow et al., 1987).

Following intra-peritoneal challenge with the ME7 scrapie strain, scrapie infectivity is detectable within the spleen 35 days after inoculation and reaches plateau levels by 70 days after inoculation (Brown et al., 1999; Fraser, 1996; Mabbott et al., 2000b) Similar targeting and accumulation of scrapie infectivity in the spleen is observed following skin scarification. Data presented here demonstrates that scrapie infectivity is detectable within the spleen at 42 days after inoculation via the skin and plateaus at approximately 63 days after inoculation. The importance of the spleen for the accumulation of scrapie infectivity following peripheral challenge has been demonstrated by splenectomy and genetic asplenia of rodent models either prior to or shortly after challenge (Clarke and Haig, 1971; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978). Splenectomy or genetic asplenia of scrapie infected rodent models significantly extends survival time (Clarke and Haig, 1971; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978). However, the presence of the spleen is not obligate to establishing scrapie infection as splenectomy of rodent models does not affect disease susceptibility, suggesting that other tissues are able to sustain accumulation of the scrapie agent in the absence of the spleen. Furthermore, splenectomy of mice either shortly before or after subcutaneous inoculation with the scrapie agent has no affect on the disease incubation period (Fraser et al., 1992; Kimberlin and Walker, 1989a). In this study scrapie infectivity accumulated to high levels within the draining ILN prior to its detection within the spleen, suggesting that

the spleen is likewise unlikely to play an important role in the accumulation of the agent following inoculation via the skin.

Data presented here demonstrates that scrapie infectivity does not replicate in the skin following skin scarification but first accumulates in the draining ILN between 24 hrs and 7 days after inoculation. Scrapie infectivity accumulates to high levels within the draining ILN prior to spreading to other lymphoid tissues. These data also suggest that scrapie infectivity is transported from the draining ILN to other lymphoid tissues via the blood due to the similar time course of targeting of infectivity to the left ILN and spleen after inoculation. Furthermore, the spleen is unlikely to play a critical role in scrapie pathogenesis after inoculation via the skin as scrapie infectivity was not detected within the spleen until 42 days after inoculation, a time point at which infectivity had already accumulated to high levels within the draining ILN. In conclusion, high levels of scrapie infectivity accumulate in lymphoid tissues during the first few weeks after inoculation by skin scarification.

4

Accumulation of the scrapie agent in lymphoid tissues after exposure through the skin is dependent on follicular dendritic cells

4.1 Abstract

Previous studies in this thesis (Chapter 3) have shown that following inoculation via the skin, scrapie infectivity accumulates within lymphoid tissues. Furthermore, studies by others have suggested that this accumulation within lymphoid tissues is critical for the transmission of the scrapie agent to the brain, as severe combined immunodeficiency mice are refractory to infection. Until now, it was not known which components of the immune system are required for efficient neuroinvasion of the scrapie agent after skin scarification. A chimeric mouse model was utilised which had a mismatch in PrP status between FDCs and other bone-marrow derived cells. This mouse model was produced by grafting of SCID/*Prnp*$^{+/+}$ mice with either PrP expressing (*Prnp*$^{+/+}$) or PrP deficient (*Prnp*$^{-/-}$) bone-marrow from immunocompetent mice. The resulting mismatch in PrP status between cell populations allowed for the identification of the cell population that was critical for the accumulation of the scrapie agent within lymphoid tissues. Here it is demonstrated that mature follicular dendritic cells are essential for the accumulation of PrP^{Sc} and infectivity within lymphoid tissues and the subsequent transmission of the scrapie agent to the brain. Furthermore, the accumulation of PrP^{Sc} and infectivity in the spleen is independent of the PrP status of lymphocytes or other bone-marrow-derived cells.

4.2 Introduction

In many TSE diseases, following peripheral inoculation, high levels of infectivity and PrP^{Sc} usually accumulate in lymphoid tissues prior to the dissemination of infection to the central nervous system (CNS). Within the lymphoid tissues of TSE infected hosts, disease-specific PrP accumulation initially takes place within germinal centres in association with follicular dendritic cells (FDCs) (Hill et al., 1999; Jeffrey et al., 2000b; Mabbott et al., 2000b; McBride et al., 1992; Sigurdson et al., 1999; van Keulen et al., 1996). Studies in rodents inoculated intra-peritoneally with the scrapie agent have shown that mature FDCs are critical for accumulation of the scrapie agent in lymphoid tissues (Brown et al., 1999; Fraser et al., 1996; Mabbott et al., 2000a; Mabbott et al., 2002; Mabbott et al., 2000b). Furthermore, in the absence of mature FDCs, the spread of disease to the central nervous system (CNS) is significantly impaired (Brown et al., 1999; Fraser et al., 1996; Klein et al., 1998; Mabbott et al., 2000a; Mabbott et al., 2002; Mabbott et al., 2000b; Montrasio et al., 2000; Prinz et al., 2002).

Previous studies in this thesis have shown that following inoculation via the skin, scrapie infectivity accumulates within lymphoid tissues (Chapter 3). Taylor *et al* (1996) also suggested that a functional immune system is critical for the translocation of the scrapie agent to the CNS following skin scarification, as severe combined immunodeficiency (SCID) mice, which lack functional T-lymphocytes, B-lymphocytes and mature FDC networks, are refractory to scrapie infection by this route of inoculation (Taylor et al., 1996a). These studies also demonstrated that after skin scarification, neuroinvasion does not occur from the skin via direct

haematogenous spread or by direct uptake by peripheral nerves. The components of the immune system that are required for efficient neuroinvasion by this route are not known. However candidate cells within the lymphoid tissues include FDCs, which are critical for the efficient transmission of the disease to the CNS following other peripheral routes of inoculation (Brown et al., 1999; Fraser et al., 1996; Klein et al., 1998; Mabbott et al., 2000a; Mabbott et al., 2002; Mabbott et al., 2000b; Montrasio et al., 2000; Prinz et al., 2002).

To maintain TSE infection, host cells must express the cellular isomer of the host prion protein, as mice deficient in PrP^C (*Prnp*$^{-/-}$ mice) do not develop disease (Bueler et al., 1993; Manson et al., 1994a). Thus, in order to determine which cells are critical for the efficient transmission of the scrapie agent to the CNS after inoculation via the skin, a chimeric mouse model was used that had a mismatch in PrP^C status between FDCs and other bone-marrow derived cells within lymphatic tissues (Brown et al., 1999). These chimeric mice were inoculated with the scrapie agent by skin scarification to allow the separate roles of FDCs and lymphocytes in peripheral scrapie pathogenesis to be determined.

4.3 Results

4.3.1 Bone-marrow reconstitution of SCID/*Prnp*$^{+/+}$ mice restores scrapie susceptibility after inoculation by skin scarification

To test the hypothesis that mature FDCs are critical for the translocation of the scrapie agent to the CNS after skin scarification, a chimeric mouse model was used which had a mismatch in PrP status between its FDCs and lymphocyte populations (Brown et al., 1999). The mouse models were produced by grafting SCID/*Prnp*$^{+/+}$ mice with either PrP expressing (*Prnp*$^{+/+}$) or PrP-deficient (*Prnp*$^{-/-}$) bone-marrow (BM) from immunocompetent 129/Ola mice (Chapter 2; section 2.3.1). As FDCs (in contrast to lymphocytes) are not considered to be derived from BM in adult mice (Kaspasi et al., 1998; Tkachuk et al., 1998), the lymphoid tissues of SCID/*Prnp*$^{+/+}$ mice grafted with *Prnp*$^{-/-}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM) have PrP expressing FDCs and other stromal derived cells, but lack PrP expression on lymphocytes. In contrast SCID/*Prnp*$^{+/+}$ mice grafted with *Prnp*$^{+/+}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) contain both PrP expressing FDCs and lymphocytes.

Twenty-eight days after BM grafting, wild-type 129/Ola mice, SCID/*Prnp*$^{+/+}$ mice, SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM mice and SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice were inoculated with the ME7 scrapie strain by skin scarification. All immunocompetent wild-type 129/Ola mice developed clinical signs of scrapie approximately 333 ± 13 days post-inoculation (n = 12; Fig. 4.1). Characteristic disease-specific PrP accumulation (Fig. 4.2, panel a), spongiform pathology (Fig.4.2, panel e) and pathological targeting of vacuolation (Fig. 4.3) typical of a peripheral infection with the ME7 scrapie strain was detected in the brains of all wild-type mice which

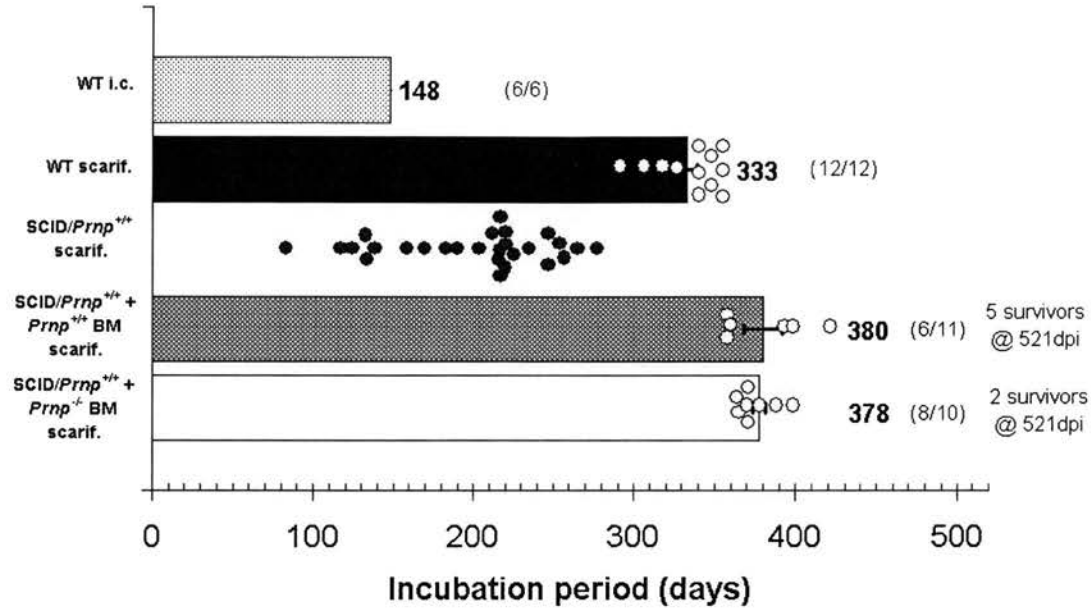


Figure 4.1- Reconstitution of SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM restores susceptibility to scrapie when inoculated by skin scarification. Wild-type (WT) mice (▮), SCID/*Prnp*$^{+/+}$ mice, SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{+/+}$ BM (▩; SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) and SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{-/-}$ BM (□; SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM) were inoculated with the ME7 scrapie strain by skin scarification (scarif.). Wild-type mice were also inoculated by i.c. injection as a titre control (▨; WT i.c). Each bar represents the mean incubation period ± S.E.M. (o) Incubation periods for individual mice that succumbed to clinical scrapie. (•) Times at which SCID/*Prnp*$^{+/+}$ mice succumbed to non-TSE related disease.

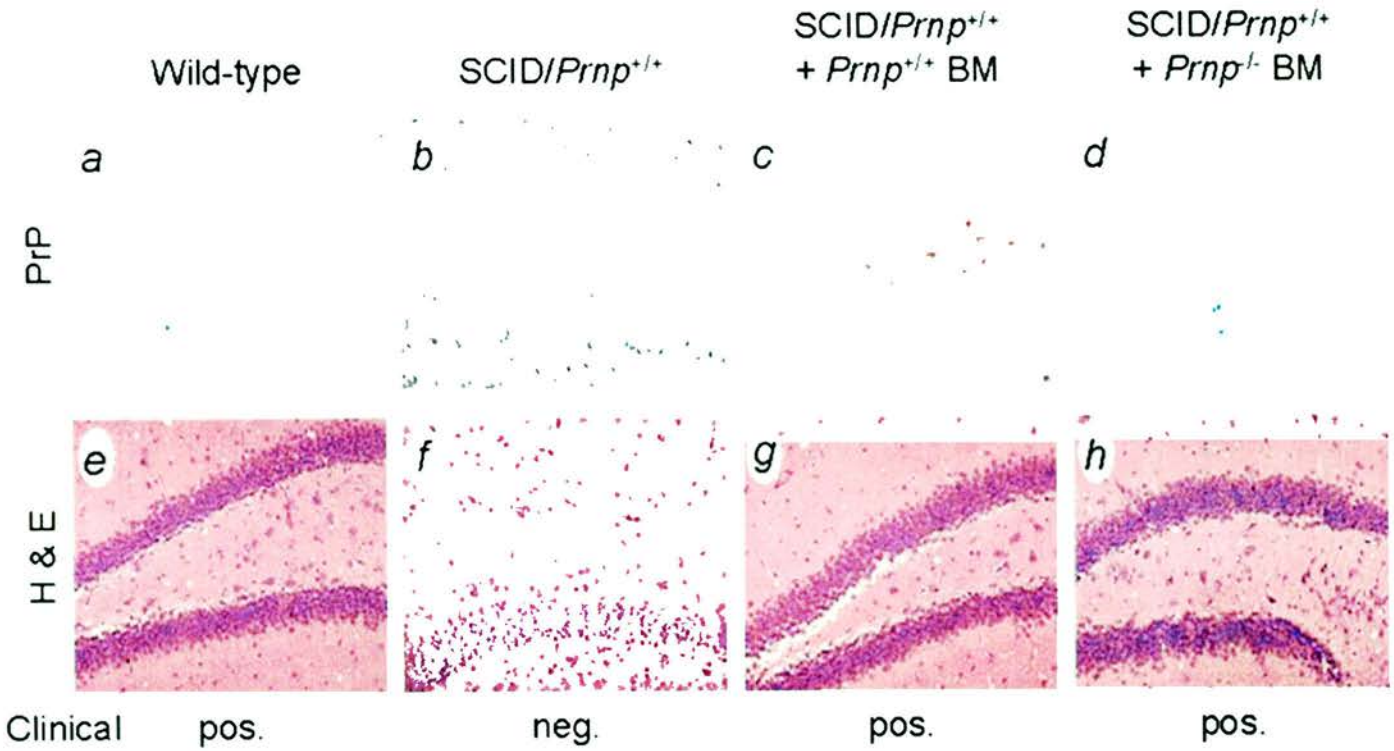
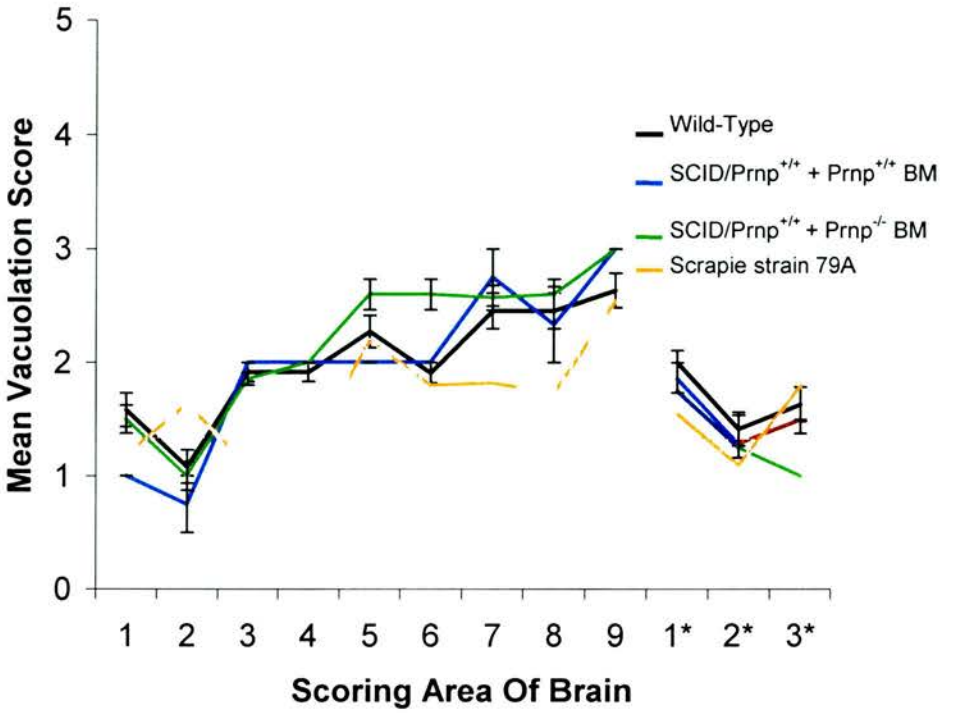


Figure 4.2- Histological analysis of brain tissue from wild-type mice (a and e), SCID/*Prnp*$^{+/+}$ mice (b and f), SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{+/+}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM; c and g), and SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{-/-}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM; d and h) inoculated with scrapie by skin scarification. Large PrP accumulations (brown; upper row) and spongiform pathology (H & E; lower row) were detected in the hippocampi of all mice which developed the clinical signs of scrapie. In contrast, no evidence of PrP accumulation (b) or spongiform pathology (f) was detected in the brains of any SCID/*Prnp*$^{+/+}$ mice that succumbed to non-TSE diseases up to 274 dpi. All sections were counterstained with haematoxylin (blue). pos., mice that developed clinical signs of scrapie; neg., mice that were free of the signs of scrapie. Original magnification X 200.



SCORING AREA	Anatomical Location in the Brain
G1	Medulla
G2	Cerebella lobule 1
G3	Superior colliculus
G4	Hypothalamus
G5	Thalamus
G6	Hippocampus
G7	Septum
G8	Retrosplenial cortex
G9	Secondary motor and cingulate cortex
W1*	Inferior and middle cerebellar peduncles
W2*	Decoction of superior cerebella peduncles
W3*	Cerebral peduncles

Figure 4.3- Reconstitution of SCID/*Prnp*$^{+/+}$ mice with immunocompetent bone marrow (BM) does not significantly affect the pathological targeting of vacuolation in the brain when compared to the vacuolation observed in scrapie challenged wild-type mice. Wild-type mice (black trace), SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM mice (blue trace) and SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice (green trace) were inoculated with ME7 scrapie strain by skin scarification. Each point represents the mean vacuolation score ± S.E.M. for each experimental group of mice within a particular scoring area of the brain. The lesion profile of C57BL mice inoculated with scrapie strain 79A is shown for comparison (orange trace).

developed clinical disease. In contrast, previous studies have shown that SCID mice are refractory to challenge with the ME7 scrapie strain by skin scarification up to 586 days post-inoculation (Taylor et al., 1996a). Unfortunately, despite careful husbandry, all ungrafted immunodeficient SCID/*Prnp*$^{+/+}$ mice in this study succumbed to non-TSE diseases up to 274 days post-inoculation (Fig. 4.1). Immunohistochemical analysis of brain tissue from all SCID/*Prnp*$^{+/+}$ mice inoculated with the scrapie agent failed to detect any signs of disease-specific PrP accumulation (Fig. 4.2, panel b) or spongiform pathology (Fig. 4.2, panel f) consistent with the hypothesis that these mice are refractory to scrapie infection following peripheral inoculation. However, the susceptibility of most SCID/*Prnp*$^{+/+}$ mice to scrapie infection was restored after grafting with, *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ BM (Fig. 4.1). Here 6/11 SCID/*Prnp*$^{+/+}$ mice grafted with *Prnp*$^{+/+}$ BM developed scrapie with a mean incubation period of 380 ± 12 days post-inoculation. Likewise 8/10 SCID/*Prnp*$^{+/+}$ mice grafted with *Prnp*$^{-/-}$ BM developed scrapie with a mean incubation period of 378 ± 4 days post-inoculation. Characteristic spongiform pathology (Fig. 4.2, panels g and h respectively) and disease-specific PrP accumulation (Fig. 4.2, panels c and d respectively) was detected in the brains of all grafted SCID/*Prnp*$^{+/+}$ mice that succumbed to clinical disease.

No significant difference was observed between the mean disease incubation periods of SCID/*Prnp*$^{+/+}$ mice grafted with either *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ BM. However, a significant statistical difference was observed between the mean incubation periods of SCID/*Prnp*$^{+/+}$ mice grafted with either *Prnp*$^{+/+}$ BM or *Prnp*$^{-/-}$ BM when compared to wild-type mice (P = 0.014 and P = 0.004 respectively). Grafted SCID/*Prnp*$^{+/+}$

mice developed clinical scrapie approximately 47 days later than the mean incubation period of immunocompetent wild-type controls (Fig. 4.1). However, no significant difference in the pathological targeting of vacuolation in the brain was observed between wild-type and grafted SCID/*Prnp*$^{+/+}$ mice (Fig. 4.3) suggesting neuroinvasion had occurred via a common pathway in each case.

4.3.2 Confirmation of immune status and germinal centre architecture

Spleens and sera were taken from all mice to monitor immune status. Consistent with the absence of mature B-lymphocytes in SCID mice (Bosma et al., 1983; Kapasi et al., 1993), serum from all ungrafted SCID/*Prnp*$^{+/+}$ mice contained undetectable levels of immunoglobulin (Ig) (mean OD 490 nm = 0.05 ; approximately ≤10 ng/ml; Fig 4.4) when compared with those of wild-type mice (mean OD 490 nm = 1.78 ;approximately ≥ 1mg/ml; Fig 4.4). Therefore there is at least a 1000 fold difference in Ig content between these two groups. However, ELISA analysis confirmed that reconstitution of SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM from either *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ mice restored serum Ig levels to those observed in wild-type mice (mean OD 490 nm = 1.81 and 1.68 respectively; approximately ≥1mg/ml; Fig 4.4). Thus functional BM derived B-lymphocytes had been successfully grafted into recipient SCID/*Prnp*$^{+/+}$ mice.

Next the *Prnp* genotype in the spleens of grafted mice was determined by PCR analysis of total splenic DNA (Fig. 4.5). Analysis of DNA from SCID/*Prnp*$^{+/+}$ mice grafted with *Prnp*$^{+/+}$ BM detected the presence of only the *Prnp* gene by the visualisation of a single band at 750 bp (Fig. 4.5 a, lanes 1-4). In contrast, two bands

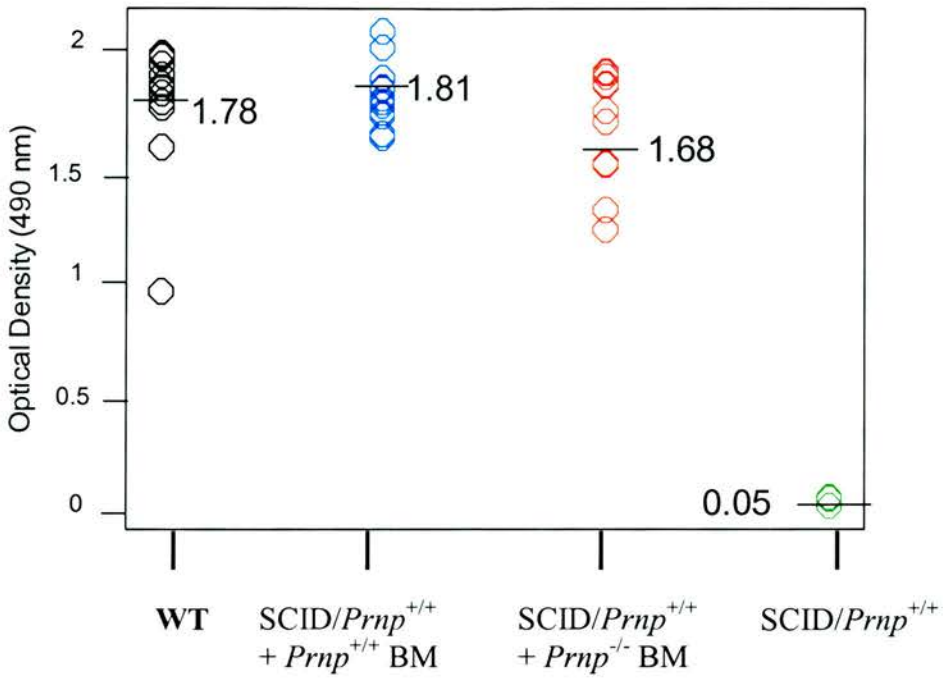


Figure 4.4- Reconstitution of SCID/*Prnp*$^{+/+}$ mice with immunocompentent bone marrow restores serum immunoglobulin levels. Serum samples from wild-type (WT) (O), SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{+/+}$ bone marrow (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) (O), SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{-/-}$ bone marrow (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM) (O) and SCID/*Prnp*$^{+/+}$ mice (O) were diluted 1/1000 and total immunoglobulin levels determined by ELISA. Samples were assayed in duplicate and each circle represents the mean optical density (OD) at 490 nm for individual mice. —, Represents mean OD at 490 nm for each experimental group of mice.

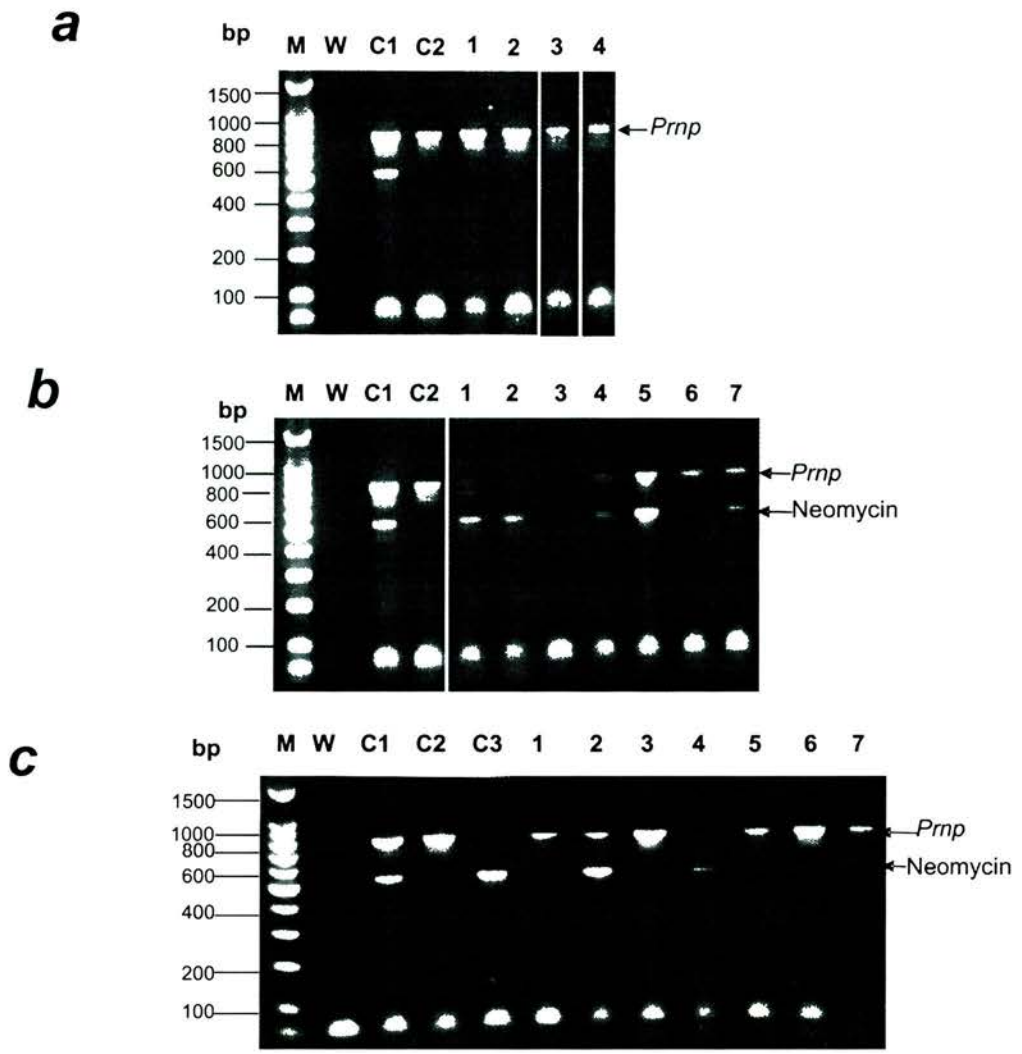


Figure 4.5- Confirmation of the *Prnp* genotype in the spleens of terminally scrapie affected, (a) SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{+/+}$ bone marrow (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) and (b) SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{-/-}$ bone marrow (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM), was determined by PCR analysis of total splenic DNA. All products were resolved through gel electrophoresis containing ethidium bromide. (a) PCR analysis of DNA from SCID/*Prnp*$^{+/+}$ mice + *Prnp*$^{+/+}$ BM confirmed the presence of the *Prnp* gene by the visualisation of a single band at 750 bp (Lanes 1-4). (b)The visualisation of two bands at 750 bp and 550 bp (Lanes 1, 2, 4-7) confirmed the presence of both *Prnp* gene and a portion of the neomycin gene respectively within DNA from SCID/*Prnp*$^{+/+}$ mice + *Prnp*$^{-/-}$ BM. (c) Determination of *Prnp* genotype of grafted SCID/*Prnp*$^{+/+}$ mice, which remained free from the signs of scrapie 521 days post-inoculation. Lane M, 100 bp molecular size marker. Controls included C1= Splenic DNA from a SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mouse, C2= Splenic DNA from wild-type/*Prnp*$^{+/+}$ mouse and C3= Splenic DNA from a *Prnp*$^{-/-}$ deficient mouse. (W) PCR amplified water was used as a negative control.

were detected in splenic DNA samples from SCID/*Prnp*$^{+/+}$ mice grafted with *Prnp*$^{-/-}$ BM, demonstrating the presence of both the *Prnp* gene (750 bp) and the neomycin resistance gene (550 bp) (Fig. 4.5 b, lanes 1-2, 4-7). Thus, these results confirmed the presence of only *Prnp*$^{+/+}$ cells within the spleens of SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM mice, and the presence of both *Prnp*$^{+/+}$ and *Prnp*$^{-/-}$ cells within the spleens of SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice.

Germinal centre architecture in the spleen was analysed by immunohistochemistry. As expected, FDC-M2 and CD35 expressing FDC networks and B-lymphocytes (CD45R/B220) were detected in the spleens of all immunocompetent wild-type mice (Fig. 4.6, panels a, e and i respectively). In contrast, both FDC networks and B-lymphocytes were absent in spleens of ungrafted SCID/*Prnp*$^{+/+}$ mice (Fig. 4.6, panels b, f and j respectively), consistent with the previously described immunodeficent phenotype of SCID mice (Bosma et al., 1983; Kapasi et al., 1993). However, FDC networks and B-lymphocytes were restored in spleens of SCID/*Prnp*$^{+/+}$ mice after grafting with either *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ BM (Fig. 4.6, panels c, g and k and Fig. 4.6, panels d, h and l respectively). Thus, the restoration of germinal centre architecture in the lymphoid tissues of SCID/*Prnp*$^{+/+}$ mice after BM grafting coincided with the restored susceptibility of these mice to scrapie infection.

4.3.3 Scrapie infectivity and PrP^{Sc} accumulation in the spleen

After skin scarification with the ME7 scrapie strain, high levels of infectivity accumulate in the spleen within from 42 days post-inoculation and are maintained throughout the course of infection (Fig 3.1; Chapter 3). In this study spleen samples

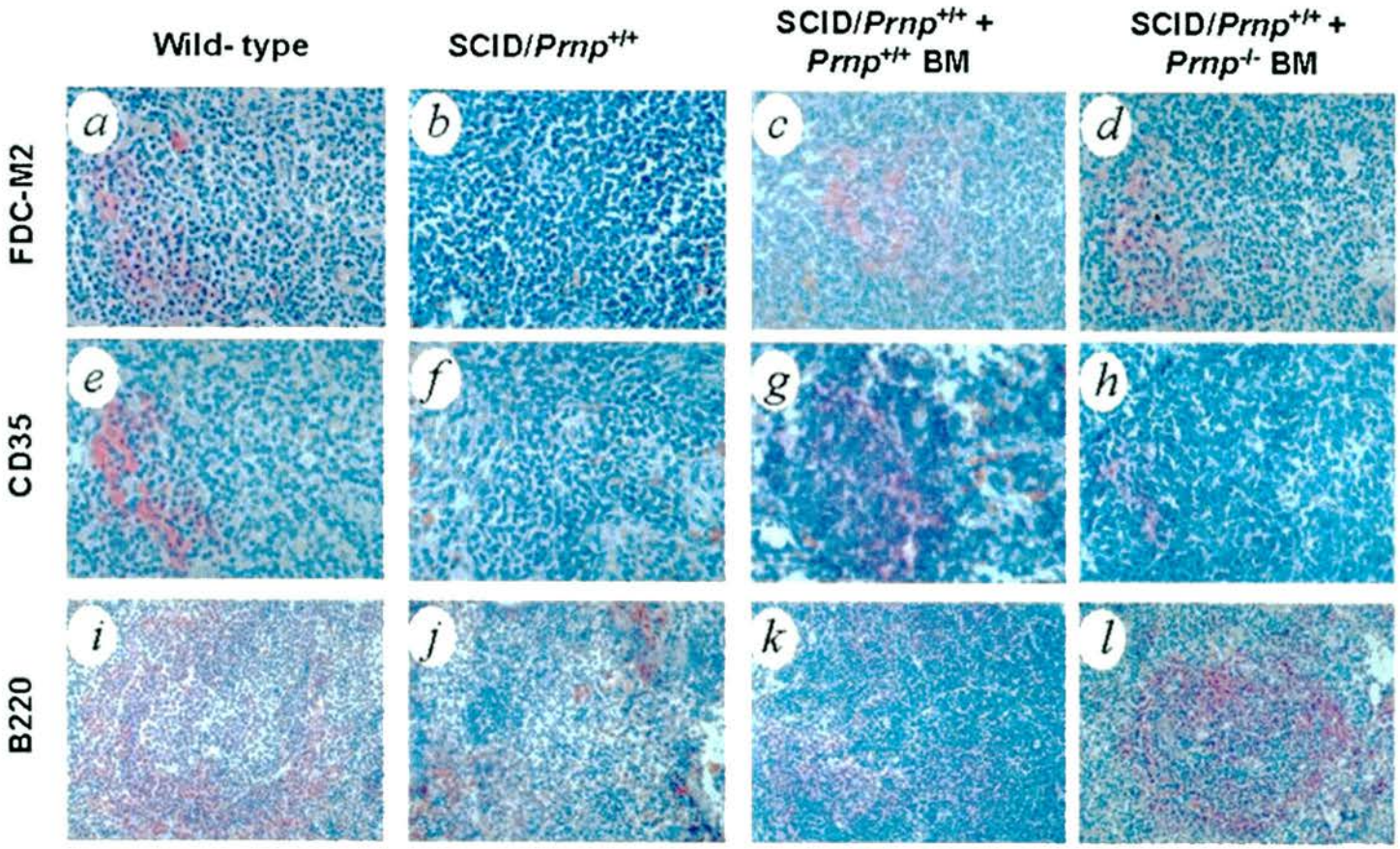


Figure 4.6- Immunohistochemical analysis of germinal centre structure in spleen tissue from terminally scrapie affected mice. Sections from wild-type (a), SCID/*Prnp*$^{+/+}$ mice (b), SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{+/+}$ bone marrow (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) (c) and SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{-/-}$ bone marrow (SCID/*Prnp*$^{+/+}$+ *Prnp*$^{-/-}$ BM) (d) were stained with FDC-M2 antiserum to detect FDCs (red). (e-h) Adjacent sections from wild-type mice (e), SCID/*Prnp*$^{+/+}$ mice (f), SCID/*Prnp*$^{+/+}$mice + *Prnp*$^{+/+}$ BM (g) and SCID/*Prnp*$^{+/+}$ mice + *Prnp*$^{-/-}$ BM (h) were stained with the CD35-specific antiserum to detect complement receptor 1 (red). (i-l) Adjacent sections from wild-type mice (i), SCID/*Prnp*$^{+/+}$ mice (j), SCID/*Prnp*$^{+/+}$ mice + *Prnp*$^{+/+}$ BM (k) and SCID/*Prnp*$^{+/+}$ mice + *Prnp*$^{-/-}$ BM (l) were stained with the CD45R-specific antiserum B220 to detect B-lymphocytes (red). All sections were counterstained with hematoxylin (blue). Brown staining in panels b, f, g, j is due to the presence of haemosiderin. Original magnification (a-h) X400, (i-l) X200.

were taken from four mice from each experimental group of mice 220 days post-inoculation. The scrapie infectivity titre in spleen lysates from each group was estimated by bioassay in groups of up to 12 indicator mice. As expected, spleens from wild-type mice inoculated with the scrapie agent contained high levels of infectivity (approximately 5.7 log i.c. ID_{50}/g). In contrast, scrapie infectivity was undetectable in spleen samples from ungrafted SCID/*Prnp*$^{+/+}$ mice assayed 220 days post-inoculation suggesting a scrapie infectivity titre, if present, below 2.7 log i.c. ID_{50}/g (at least 1000 fold less than the level detected in spleens of wild-type mice assayed at the same time post inoculation). Scrapie infectivity accumulation was restored in the spleens of SCID/*Prnp*$^{+/+}$ mice after grafting with either *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ BM to the same magnitude observed in wild type mice at the same time point (approximately 6.7 and 5.7 log i.c. ID_{50}/g, respectively).

Similarly, immunoblot analysis of spleen tissue from terminally scrapie-affected wild-type mice detected large accumulations of detergent-insoluble proteinase-K resistant PrP^{Sc} (Fig. 4.7 a, lane 2 and Fig. 4.7 b, lanes 2 and 4). A typical three-banded pattern was observed between molecular mass values of 20-30 kDa, representing the unglycosylated, monoglycosylated and diglycosylated isomers of PrP (in order of increasing molecular mass). However, no PrP^{Sc} accumulation was detectable within the spleens of any ungrafted SCID/*Prnp*$^{+/+}$ mice assayed at various time points after inoculation (Fig. 4.7 a, lanes 4, 6 and 8). In comparison, PrP^{Sc} accumulation was restored in the spleens of terminally scrapie-affected SCID/*Prnp*$^{+/+}$ mice grafted with either *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ BM to levels similar to those observed in wild type mice (Fig. 4.7 b, lanes 7, 9 and 11).

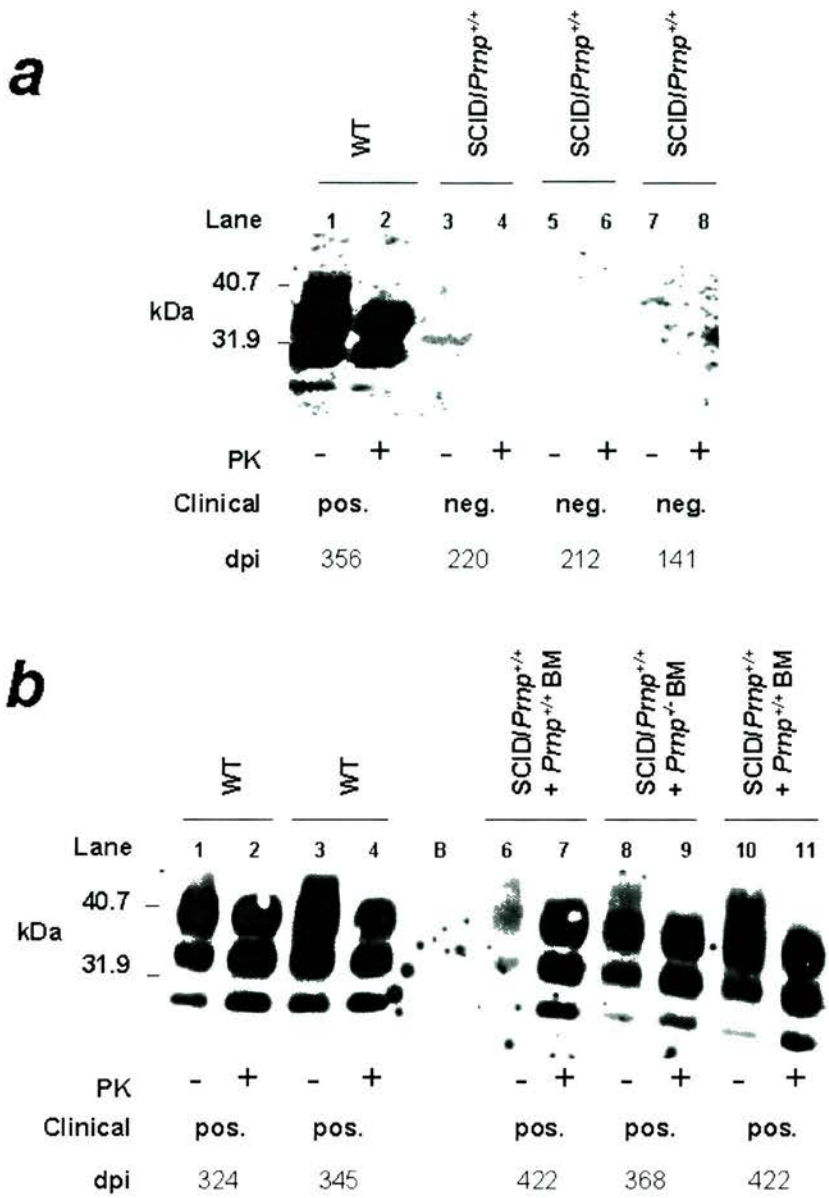


Figure 4.7- Immunoblot analysis of spleen tissue from wild-type mice (WT), SCID/*Prnp*$^{+/+}$ mice, SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{+/+}$ bone marrow (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) and SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{-/-}$ bone marrow (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM) following skin scarification with scrapie strain ME7. Samples were treated in the absence (-) or presence (+) of proteinase K prior to electrophoresis. (a) High levels of PrP^{Sc} accumulation were detected in the spleens of terminally scrapie affected WT mice but none was detected in the tissues SCID/*Prnp*$^{+/+}$ mice at any time point. (b) However, high levels of PrP^{Sc} were detected in spleens of SCID/*Prnp*$^{+/+}$ mice grafted with either *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM or SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice respectively) Lane B is blank. pos., mice that developed clinical signs of scrapie; neg., mice that were free from the signs of scrapie; dpi., days post-inoculation at which the tissues were analysed.

4.3.4 Reconstituted SCID/*Prnp*$^{+/+}$ mice which did not succumb to scrapie infection

Although the grafting of most SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM restored susceptibility to scrapie, 5/11 SCID/*Prnp*$^{+/+}$+ *Prnp*$^{+/+}$ BM mice and 2/10 SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice remained free from the signs of scrapie up to 521 days post-inoculation, at which point the experiment was terminated (Fig. 4.1). Immunohistochemical analysis of brain tissue from all surviving mice failed to detect any spongiform change (Fig. 4.8, panels c and d) or PrP^{Sc} accumulation (Fig. 4.8, panels a and b). Similarly no PrP^{Sc} was detected, by immunoblot analysis, in the spleens from these surviving mice, taken 521 days post-inoculation (Fig. 4.9 lanes 4, 6 and 8). Taken together these data suggest these mice would have not developed clinical scrapie at a later stage.

The immune status of all surviving mice was determined by measuring serum Ig levels by ELISA and the presence of grafted cells in the spleen by PCR analysis. The serum Ig levels of each of the five SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM mice (mean OD 490 nm = 1.81; approximately $\geq$ 1mg/ml; Fig 4.4) and the two SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$BM mice (mean OD = 1.68; approximately $\geq$ 1mg/ml; Fig 4.4) were similar to the serum Ig levels displayed by the wild-type mice, suggesting these mice had been successfully grafted and functional B-lymphocytes were present. Next the *Prnp* genotype in the spleens of the grafted surviving SCID/*Prnp*$^{+/+}$ was determined by PCR analysis. PCR analysis of DNA from the five surviving SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM mice confirmed the presence of the *Prnp* gene (750 bp) (Fig. 4.5 c, lanes 1, 3, 5-7). Furthermore, bands representing the *Prnp* gene (750 bp) and the

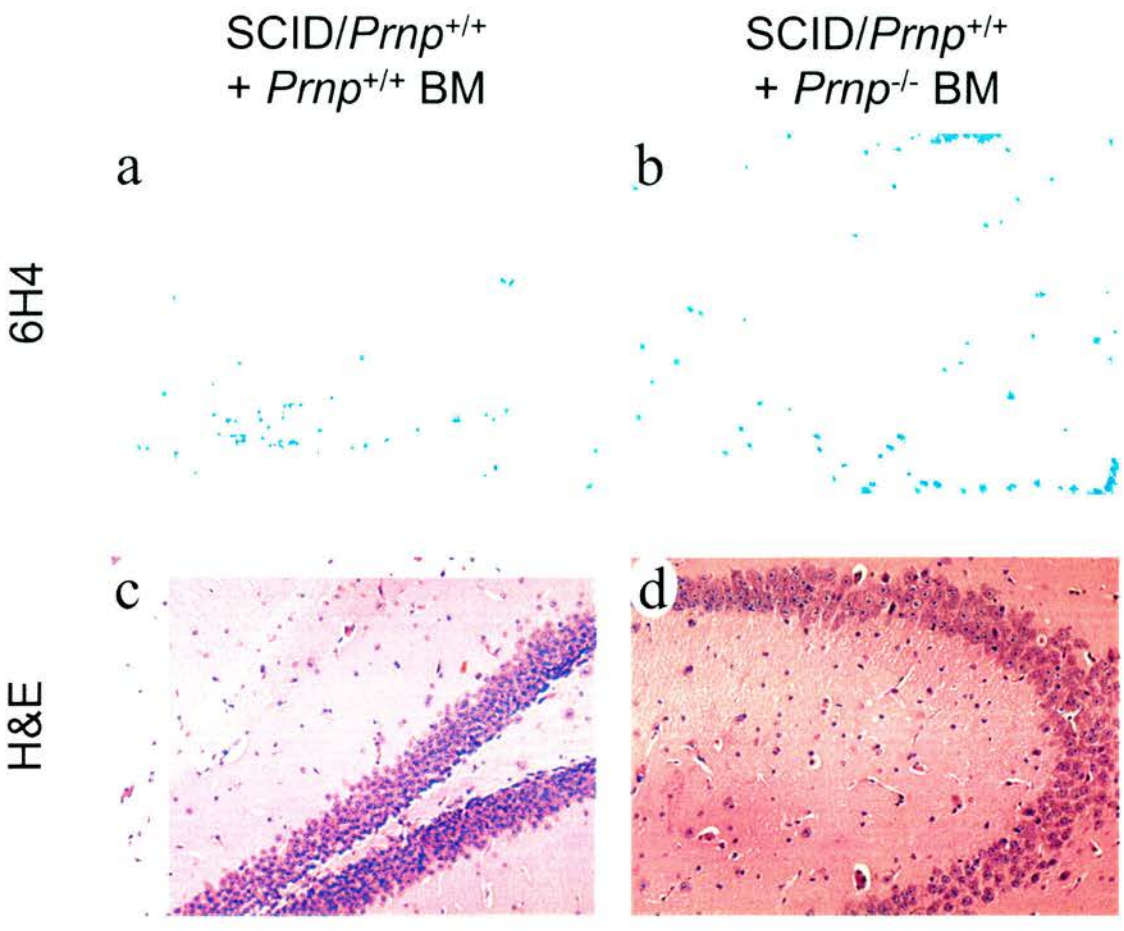


Figure 4.8- Histological analysis of brain tissue from SCID/*Prnp*$^{+/+}$ mice reconstituted with either *Prnp*$^{+/+}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) (a and c) or *Prnp*$^{-/-}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM) (b and d) that remained free of the signs of scrapie 521 days post-inoculation. (a and b) No PrP accumulations (brown) were detected in the hippocampus. PrP was detected with the PrP-specific monoclonal antiserum 6H4 and sections were counterstained with hematoxylin (blue). (c and d) Adjacent sections were stained with hematoxylin and eosin (H&E) and no TSE-specific vacuolation was detected. Original magnification X200.

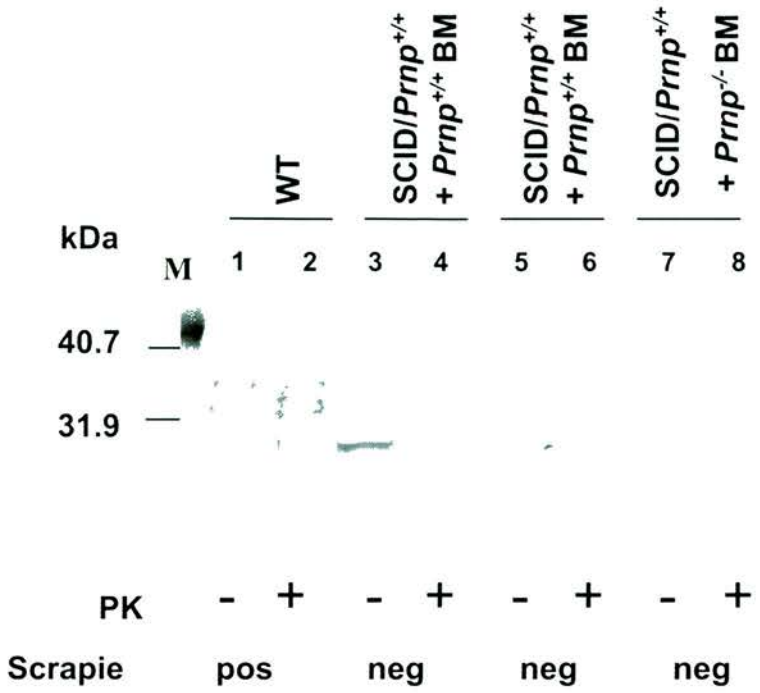


Figure 4.9- Immunoblot analysis of spleen tissue from terminally scrapie infected wild-type mice (WT), SCID/*Prnp*$^{+/+}$ mice reconstituted with either *Prnp*$^{+/+}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) or *Prnp*$^{-/-}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM) which remained free of signs of scrapie 521 days post-inoculation, following scarification with scrapie strain ME7. Samples were treated in the presence (+) or absence (-) of proteinase K (PK) prior to electrophoresis. PrP^{Sc} accumulation was detected within the spleens of terminally scrapie affected WT mice, however no PrP^{Sc} accumulation could be detected within the scrapie negative SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM mice or SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice. Lane M contains a molecular size marker.

neomycin resistance gene (550 bp) were detected within the spleens of the two surviving SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice (Fig. 4.5c, lanes 2 and 4), confirming that there had been a successful introduction of *Prnp*$^{-/-}$ bone-marrow derived cells into these mice.

4.4 Discussion

Previous studies in mice have shown that skin scarification is an effective means of scrapie transmission in immunocompetent mice (Taylor et al., 1996a). However highly immunodeficient SCID mice are refractory to scrapie infection following inoculation via this route, suggesting that a functional immune system is critical to the transmission of the scrapie agent to the CNS after inoculation via the skin (Taylor et al., 1996a). In this study it has been demonstrated that reconstitution of SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM restores scrapie susceptibility after skin scarification. This effect also coincides with the restoration of mature FDC networks within the spleens of grafted SCID/*Prnp*$^{+/+}$ mice. Furthermore it has been shown that scrapie transmission to the CNS is independent of the PrP status of lymphocytes and other BM derived cells. Taken together these findings are consistent with previous studies, which have demonstrated that after peripheral exposure with scrapie strain ME7, a functional immune system, and more critically PrP-expressing FDCs, are required for transmission of the agent to the CNS (Brown et al., 1999; Farquhar et al., 1994; Kimberlin and Walker, 1979; Mabbott et al., 2000a; Mabbott et al., 2000b).

SCID mice suffer from a congenital syndrome that is characterized by the deficiency of both B and T- lymphocyte immunity (Bosma et al., 1983). Secondary to this defect they also lack functional FDCs as cytokine stimulation from B-lymphocytes is important for the maturation and maintenance of FDC networks (Kapasi et al., 1993). Unfortunately, despite careful husbandry, due to their dysfunctional immune system all ME7 challenged SCID/*Prnp*$^{+/+}$ mice in this study succumbed to non-TSE related

diseases up to 274 days post-inoculation (Fig. 4.1). These diseases were non-infectious (e.g. thymic tumours) and were not a reflection of the microbiological status of the husbandry conditions, which were maintained to a high standard of hygiene. Previous data from this laboratory (Taylor et al., 1996a) has shown that, in contrast to wild-type mice, SCID mice did not succumb to clinical scrapie after exposure to a similar dose of scrapie-affected brain homogenate via skin scarification (mean survival period = 442 ± 21 days post-inoculation, n = 23, range = 259-586 days). After intra-peritoneal inoculation with ME7 scrapie strain, disease-specific PrP is detected in the brain considerably before the onset of clinical signs (Farquhar et al., 1994). In this study no disease-specific PrP accumulation or vacuolation was detected within the brains of SCID/*Prnp*$^{+/+}$ mice (Fig. 4.2, panel b), supporting the hypothesis that they would not have developed clinical scrapie. Furthermore, scrapie infectivity was undetectable in the spleens of SCID/*Prnp*$^{+/+}$ mice assayed 220 days post-inoculation. Similarly immunoblot analysis of spleen tissue from SCID/*Prnp*$^{+/+}$ mice confirmed that lymphoid tissues in these animals were incapable of supporting the peripheral replication of the agent after skin scarification as no PrP^{Sc} accumulation could be detected within their spleens (Fig. 4.7 a). Although unlikely, these data does not exclude the possibility that the SCID/*Prnp*$^{+/+}$ mice in this study might have gone on to develop scrapie via a lymphoid independent route. However this would contradict studies by Taylor *et al* (Taylor et al., 1996a) which showed that after skin scarification SCID mice remained free from the signs of scrapie. Although it has been previously demonstrated that it is possible to transmit BSE by blood transfusions in sheep (Houston et al., 2000; Hunter, 2003; Hunter and Houston, 2002), these data suggest that after skin scarification, infectivity is unable to reach

the CNS directly via the blood stream or peripheral nerves within the skin, but instead reaches it indirectly via the lymphoreticular tissues.

Engraftment of SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM restored functional lymphocyte populations within the spleen. Furthermore, these lymphocytes were functional as they produced immunoglobulins and were able to stimulate FDC maturation and network formation (Bosma et al., 1983; Kapasi et al., 1993). The development of germinal centre architecture comparable to immunocompetent animals coincided with restored scrapie susceptibility and the accumulation of infectivity and PrP^{Sc} in lymphoid tissues of these mice. Thus, these data demonstrate that after inoculation through scarified skin the scrapie agent accumulates in lymphoid tissues prior to neuroinvasion, as observed with other peripheral routes of exposure (Brown et al., 1999; Fraser et al., 1996; Mabbott et al., 2000a; Mabbott et al., 2002; Mabbott et al., 2000b).

The reconstitution of SCID/*Prnp*$^{+/+}$ mice with either *Prnp*$^{+/+}$ or *Prnp*$^{-/-}$ BM did not result in any visible differences in the levels of PrP^{Sc} accumulation within the spleen or in the timing of the development of the neurological disease. These results are consistent with the demonstration that PrP-expression on FDCs, and not lymphocytes, is critical for the peripheral accumulation and subsequent neuroinvasion of ME7 scrapie (Brown et al., 1999; Klein et al., 1998; Montrasio et al., 2001). As PrP^{C} expression is a pre-requisite for the transfer of TSE infection from the periphery to the CNS (Bueler et al., 1993), the above results provide no evidence for the direct involvement of BM derived cells such as B-lymphocytes in

the replication and subsequent neuroinvasion of scrapie strain ME7. As SCID/*Prnp*$^{+/+}$ mice were not γ-irradiated prior to reconstitution a proportion of BM derived cells such as dendritic cells and macrophages in grafted mice may be of host origin (i.e. PrP expressing) and a proportion of recipient origin (PrP deficient). These data suggests that the PrP status of BM derived cells such as dendritic cells, which are a potential candidate transport mechanism of the agent to the lymphoid tissues (Chapter 7) (Aucouturier et al., 2001; Huang et al., 2002) does not appear to be critical to scrapie pathogenesis. This is consistent with previous findings by Brown and colleagues, who using a chimeric mouse model equivalent to the one used in this study, determined that after intra-peritoneal inoculation with scrapie strain ME7, PrP expressing FDCs were critical for the replication of the scrapie agent in the spleen. This replication was also independent of the PrP status of the lymphocytes and other bone-marrow derived cells (Brown et al., 1999).

Interestingly, grafted SCID/*Prnp*$^{+/+}$ mice did display significantly longer incubation periods in comparison to immunocompetent wild-type mice. Similar results have also been observed in grafted SCID mice inoculated with scrapie strain C506M3 by intra-peritoneal injection (Lasmezas et al., 1996). The reason for the delay in the onset of the neurological disease in BM-grafted SCID/*Prnp*$^{+/+}$ mice is not known, but it might be that at the time of inoculation the restoration of germinal centre functionality in these mice was incomplete. This effect could have reduced the number of potential peripheral target cells, such as FDCs, available for agent replication at the time of inoculation. In the temporary absence of FDCs at the time of inoculation both the replication of the scrapie agent in the spleen and its

subsequent neuroinvasion are significantly delayed (Mabbott et al., 2000a). To achieve efficient reconstitution it has been suggested that mice are sub-lethally γ-irradiated prior to cell transfer, to encourage full maturation of grafted bone-marrow cells (Fulop and Phillips, 1986). This procedure was not undertaken in this study as SCID mice have an increased sensitivity to γ-irradiation due a general defect in their DNA repair mechanisms which is believed to be closely linked to the *scid* mutation (Fulop and Phillips, 1990). It was a concern that γ-irradiation may have adverse affects on the architecture of the skin, blood-brain barrier or nerve-brain barrier of SCID mice, any of which may have facilitated neuroinvasion by an atypical mechanism. Although neuroinvasion was delayed in the reconstituted SCID/*Prnp*$^{+/+}$ mice, no significant difference was observed in the severity or distribution of vacuolation or disease-specific PrP accumulation within the brains of wild-type and grafted SCID/*Prnp*$^{+/+}$ mice, suggesting that neuroinvasion had occurred in these mice via a common pathway.

Although the grafting of SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM restored scrapie susceptibility in most cases, 5/11 SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM mice and 2/10 SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice remained free from the signs of scrapie 521 days post-inoculation, at which point the experiment was terminated. ELISA and PCR genotyping analysis suggested that reconstitution had been successful in these surviving mice, yet they were refractory to peripheral inoculation. It takes approximately 4-6 weeks for full restoration of BM derived cell populations and germinal centre architecture (Fulop and Phillips, 1986). As this time period may vary between individual animals it is conceivable that in this study the surviving

grafted SCID/*Prnp*$^{+/+}$ mice may not have achieved a mature, fully functional immune status, prior to inoculation. Thus, this may have prevented the replication of the scrapie agent due to a lack of functional peripheral target cells such as FDCs (Mabbott et al., 2000a). In the absence of mature FDCs at the time of inoculation it is likely that a significant amount of the inoculum is destroyed by macrophages (Beringue et al., 2000b; Carp and Callahan, 1982). This delay is again consistent with the hypothesis that scrapie infectivity is unlikely to reach the CNS from the skin by the direct translocation of infectivity by nerves within the skin or by direct transport via the bloodstream.

Data presented here demonstrates that the accumulation of the scrapie agent in the spleen following inoculation by skin scarification occurs only in the presence of mature FDCs and is independent of the PrP status of surrounding splenic lymphocytes and other bone-marrow derived cells. Furthermore, these results indicate that functionally mature FDCs are required for subsequent neuroinvasion. These data are consistent with previous research using the ME7 scrapie strain which suggests that FDCs are critical for efficient neuroinvasion following intra-peritoneal exposure (Brown et al., 1999; Mabbott et al., 2000a; Mabbott et al., 2000b). Once the TSE agent spreads to the CNS the neurodegeneration they cause is considered irreversible. The identification of an important role for FDCs in the pathogenesis of disease after skin scarification provides an opportunity for therapeutic intervention prior to neuroinvasion. The potential for FDCs in therapeutic intervention is already being investigated following other peripheral routes of exposure (Mabbott et al.,

2001; Mabbott et al., 2000a; Mabbott et al., 2002; Montrasio et al., 2000) and is investigated in this thesis (Chapter 5).

5

Follicular dendritic cell dedifferentiation reduces scrapie susceptibility after inoculation via the skin

5.1 Abstract

Previous studies in this thesis (Chapter 4) have suggested that mature PrP^{C}-expressing follicular dendritic cells (FDCs) are critical for the accumulation of scrapie infectivity and PrP^{Sc} within lymphoid tissues. FDCs require lymphotoxin signals from B-lymphocytes in order to maintain their dedifferentiated state and blockade of this signalling with a lymphotoxin β-receptor immunoglobulin (LTβR-Ig) fusion protein results in the temporary dedifferentiation of FDCs within 3 days of treatment for approximately 28 days. To further investigate the role of FDCs in scrapie pathogenesis after inoculation via skin scarification, LTβR-Ig was administered to C57BL/Dk mice either 3 days before or 14 or, 42 days after inoculation. Data presented here shows that treatment with LTβR-Ig before inoculation with the scrapie agent, blocks the early accumulation of scrapie infectivity and PrP^{Sc} within the draining inguinal lymph node (ILN) and spleen. These effects coincided with the dedifferentiation of FDCs within the ILNs and spleen within 3 days of treatment. Furthermore, LTβR-Ig treatment before inoculation with the scrapie agent significantly reduces disease susceptibility and extends the disease incubation period. Treatment with LTβR-Ig 14 days after inoculation with the scrapie agent also significantly extends the disease incubation period. Taken together these data suggest that FDCs are critical for efficient neuroinvasion of the scrapie agent after inoculation via the skin. However, treatment with LTβR-Ig at 42 days after inoculation with the scrapie agent had no significant affect on disease susceptibility or on the disease incubation period, suggesting that the spread of infectivity from FDCs to peripheral nerves occurs between 14-42 days after inoculation.

5.2 Introduction

Studies in mouse scrapie models have shown that mature PrP^C-expressing follicular dendritic cells (FDCs) are critical for the accumulation of the scrapie agent within lymphoid tissues and the subsequent neuroinvasion of the agent after either intra-peritoneal (i.p.) or oral exposure (Brown et al., 1999; Mabbott et al., 2000a; Mabbott et al., 2002; Mabbott et al., 2000b; Mabbott et al., 2003). Studies in this thesis have also suggested that mature PrP^C-expressing FDCs are critical for the accumulation of the scrapie agent within lymphoid tissues after inoculation by skin scarification (Chapter 4). FDCs therefore represent a potential therapeutic target for the intervention in peripherally acquired transmissible spongiform encephalopathies (TSEs).

FDCs require a number of different cytokine signals to maintain their development and maturation (Chaplin and Fu, 1998; Kosco-Vilbois et al., 1997). Lymphotoxin (LT) is a cytokine produced by B-lymphocytes, T-lymphocytes and natural killer cells and is expressed as a membrane bound heterotrimer ($LT\alpha_1\beta_2$) (Ware et al., 1995). $LT\alpha_1\beta_2$ mediates its signalling through the LTβ receptor (LTβR) expressed on FDCs or its precursor cells (Endres et al., 1999). Mice which are genetically deficient in LTα, LTβ or LTβR do not develop mature FDC networks (Futterer et al., 1998; Koni et al., 1997; Matsumoto et al., 1996a). Similarly, in adult mice, pre-existing FDC networks can be dedifferentiated through the pharmacological blockade of the LTβR signalling pathway (Mackay and Browning, 1998). Previous studies of i.p or orally inoculated scrapie mouse models have shown that the temporary dedifferentiation of FDCs, by treatment with the LTβR immunoglobulin-

fusion protein (LTβR-Ig; (Force et al., 1995)), blocks the early accumulation of PrP^{Sc} and scrapie infectivity in lymphoid tissues, delays subsequent neuroinvasion and significantly reduces disease susceptibility (Mabbott et al., 2000a; Mabbott et al., 2003; Montrasio et al., 2000).

Although studies in this thesis (Chapter 4) have suggested that mature PrP^{C}-expressing FDCs are critical for the accumulation of PrP^{Sc} and infectivity in lymphoid tissues after inoculation via the skin, their precise role in neuroinvasion was not fully defined. Unfortunately, SCID/*Prnp*$^{+/+}$ mice in Chapter 4 succumbed to non-TSE diseases before the expected onset of the clinical symptoms of scrapie infection. Furthermore, scrapie pathogenesis in mice expressing PrP^{C} on lymphocyte populations but not FDCs could not be investigated as SCID/*Prnp*$^{-/-}$ mice (Brown et al., 1999) were not available. Thus further experiments were performed in this chapter to answer the following questions;

1) Are FDCs required for neuroinvasion of the scrapie agent after inoculation via the skin?
2) Is disease susceptibility reduced in the absence of FDCs?
3) What is the time period of FDCs involvement prior to neuroinvasion?

In order to answer these questions, FDCs were temporarily dedifferentiated by treatment with a single i.p. injection of LTβR-Ig either before or shortly after inoculation with the scrapie agent by skin scarification and the effects on pathogenesis determined.

5.3 Results

5.3.1 Effect of LTβR-Ig treatment on FDC status

Temporary blockade of the LTβR signalling pathway was achieved by a single i.p. injection of 100 μg of LTβR-Ig (Force et al., 1995), (Chapter 2. section 2.3.3). Three days after treatment with LTβR-Ig, expression of the FDCs markers FDC-M2 (Kosco-Vilbois et al., 1997)) and CD35 (complement receptor 1) were undetectable in the lymphoid follicles of inguinal lymph nodes (ILNs; Fig. 5.1). The expression of FDCs markers FDC-M1, FDC-M2 and CD35 were also undetectable in spleen tissues taken 3 days after treatment (Fig. 5.2). The effects of LTβR-Ig treatment on FDCs are temporary and last approximately 28 days (Mabbott et al., 2003; Mackay and Browning, 1998). FDCs functionally trap and retain antigens on their surface through interactions with complement components and cellular complement receptors such as CD35 (Nielsen et al., 2000; Pepys, 1976). Here the loss of the expression of complement receptor 1 (CD35) in the lymphoid tissues of LTβR-Ig treated mice coincided with the subsequent loss of expression, complement component C4 (FDC-M2) (Fig. 5.1 and Fig. 5.2). Taken together these data suggest that within 3 days of treatment with LTβR-Ig, FDCs were dedifferentiated and had lost their ability to trap and retain antigens. Treatment of mice with 100 μg of polyclonal human immunoglobulin IgG (hu-Ig) as a control had no adverse effects on FDC status in the ILNs (Fig. 5.1) or spleen (Fig. 5.2).

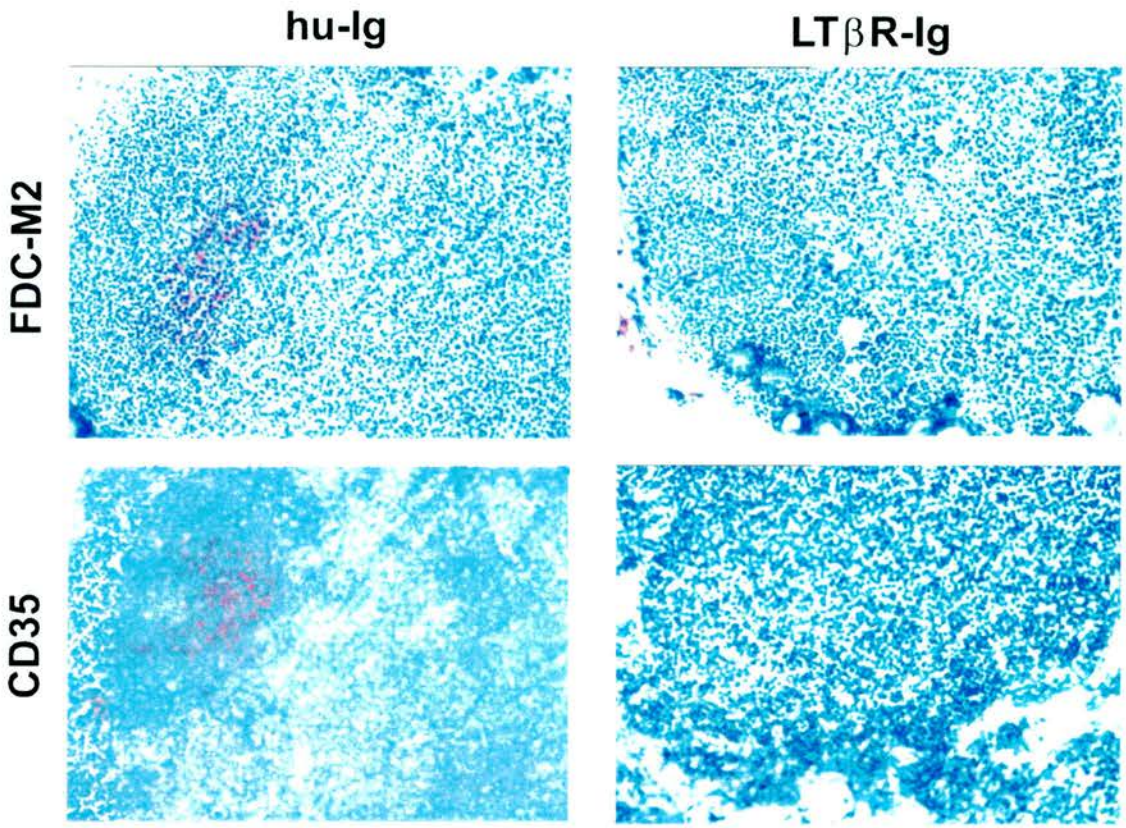


Figure 5.1- Effect of LTβR-Ig treatment on FDC status in inguinal lymph nodes of uninfected mice. Tissues were taken 3 days after injection with hu-Ig (control) or LTβR-Ig and adjacent frozen section stained with FDC-M2 (top row; red) and CD35 (bottom row; red) monoclonal antiserum to detect FDCs. FDCs were undetectable in inguinal lymph nodes 3 days after LTβR-Ig treatment. All sections were counterstained with haematoxylin (blue). Original magnification X200.

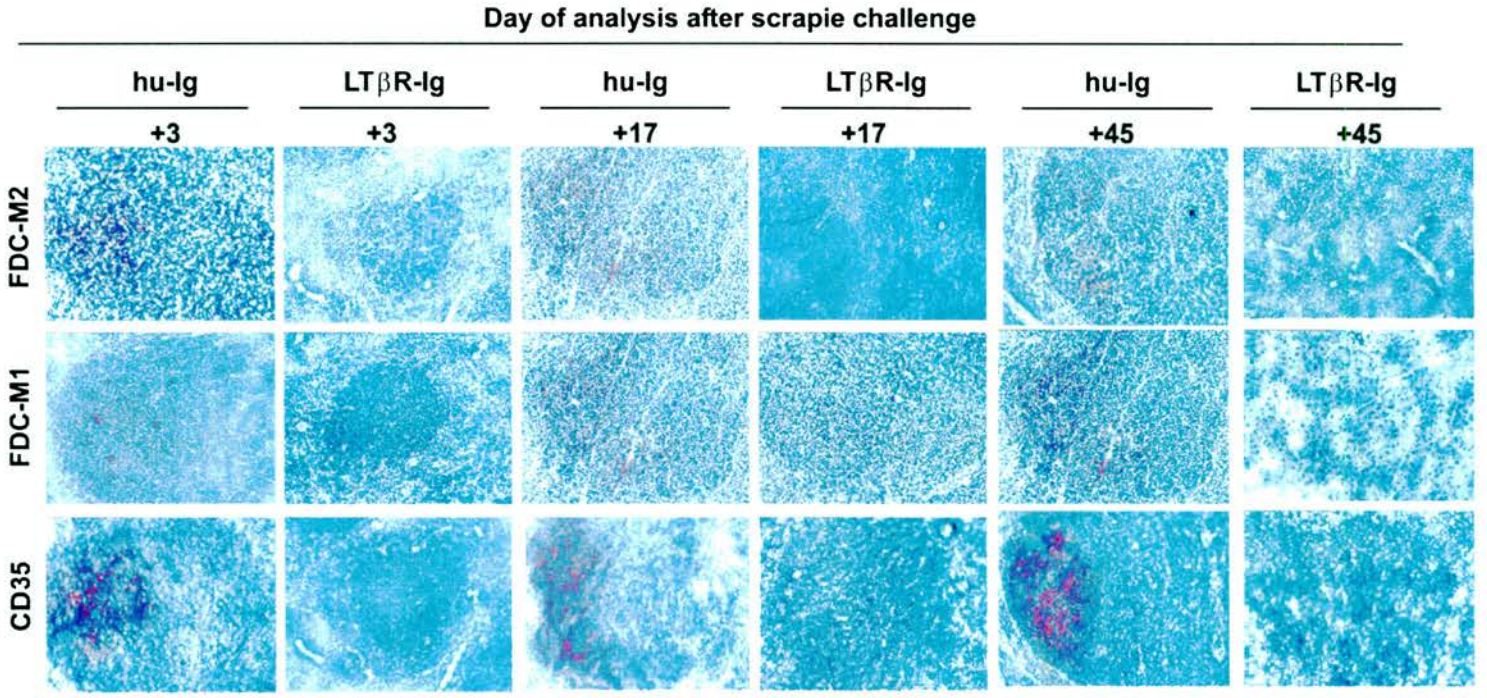


Figure 5.2- Effect of LTβR-Ig treatment on FDC status in the spleen. Tissues were taken 3 days after treatment with either hu-Ig (control) or LTβR-Ig at the times indicated post-scrapie challenge. Adjacent frozen section stained with FDC-M2 (top row; red), FDC-M1 (second row; red) and CD35 (third row; red) monoclonal antiserum to detect FDCs. FDCs were undetectable in spleens from LTβR-Ig treated mice 3 days after injection. All sections were counterstained with haematoxylin (blue). Original magnification X200.

5.3.2 Effect of LTβR-Ig treatment on the early accumulation of PrP^{Sc} within lymphoid tissues.

After inoculation of immunocompetent mice with the ME7 scrapie strain by skin scarification, high levels of infectivity accumulate in lymphoid tissues within the first few weeks post-inoculation and are maintained throughout the course of infection (Fig. 3.1; Chapter 3). Groups of mice were treated with either hu-Ig or LTβR-Ig either 14 or 42 days after inoculation with the scrapie agent by skin scarification and spleens and both ILNs were taken from two mice from each group 3 days later (day 17 and 45, respectively).

The accumulation of PrP^{Sc} within the ILNs and spleen were determined by paraffin embedded tissue (PET) immunoblot analysis ((Schulz-Schaeffer et al., 2000); Chapter 2 section 2.5.3)). PET blots shown (Table 5.1; Fig. 5.3) are representative of two mice from each treatment group and of five section analysised. Experiments demonstrated that PrP^{Sc} was detected in the right draining ILN of hu-Ig control treated mice within 17 days after inoculation. In contrast PrP^{Sc} was not detectable in the non-draining (left) ILN or the spleen from the same mice at this time point (Table. 5.1). Analysis of tissues from control treated mice 45 days after inoculation demonstrated PrP^{Sc} was present in a greater number of lymphoid follicles in the right draining ILN (Table 5.1 and Fig. 5.3). Furthermore, PrP^{Sc} was now also detectable in a single lymphoid follicle in both the left ILN and spleen (Table 5.1; Fig. 5.3). Thus these data demonstrate that PrP^{Sc} first targets the right ILN draining the site of challenge within 17 days after inoculation via the skin, and then subsequently spreads to the left ILN and spleen between 17 and 45 days post-inoculation.

Table 5.1- The effect of LTβR-Ig treatment on the early accumulation of PrP^{Sc} in inguinal lymph nodes and spleen tissue taken 3 days post-treatment [a, b].

	hu-Ig			LTβR-Ig		
Day of Treatment	Right LN[c]	Left LN[d]	Spleen	Right LN	Left LN	Spleen
14	++	-	-	+/-	-	-
42	+++	+	+	+/-	-	- /+

[a]; Mice were given a single i.p. injection (100 μg) of LTβR-Ig or hu-Ig as a control on the days indicated after inoculation with scrapie via skin scarification of the right thigh.

[b]; PrP^{Sc} accumulation in inguinal lymph nodes and spleen tissue (n = 2) was scored on the basis of the number of lymphoid follicles which immunostained positive for PrP^{Sc};

+++; 3> or more lymphoid follicles

++; 2-3 lymphoid follicles

+; one lymphoid follicles

+/-; one lymphoid follicle in only one of the samples

-; no PrP^{Sc} detected.

[c]; inguinal lymph node draining the site of inoculation

[d]; non-draining lymph node

nb- This table is representative of two mice from each group. Five serial sections from each lymph node was analysised using this method.

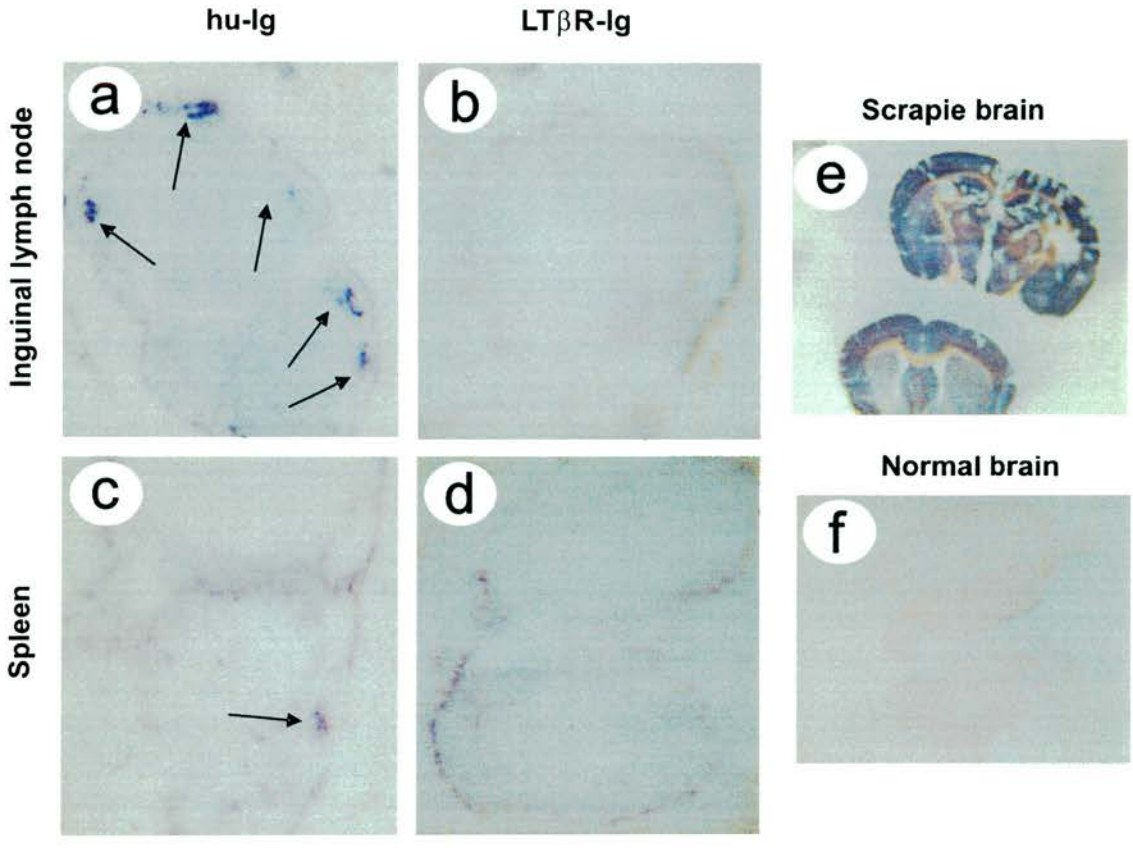


Figure 5.3- Effect of LTβR-Ig treatment on PrP^{Sc} accumulations in the right inguinal lymph node draining the site of scrapie challenge and spleen tissue 45 days after scrapie challenge. Mice received either hu-Ig (control; a and c) or LTβR-Ig (b and d) at 42 days post-inoculation and tissue samples were then collected 3 days after treatment. PrP^{Sc} accumulations (blue/purple staining) were detected in the draining inguinal lymph (a) and spleen tissue (c) of all hu-Ig treated animals. PrP^{Sc} specific staining was undetectable in all tissue samples from LTβR-Ig treated mice (b and d). Terminally scrapie affected brain tissue (e) and normal brain tissue (f) was included as controls.

When mice were treated with LTβR-Ig, the number of follicles containing PrP^{Sc} in the draining ILNs and spleen were visibly reduced or completely absent (Table 5.1 and Fig. 5.3). These data demonstrate that the temporary dedifferentiation of FDCs correlates with a rapid reduction in the number of lymphoid follicles containing PrP^{Sc} in the ILNs and spleen.

5.3.3 Effect of LTβR-Ig treatment on the accumulation of scrapie infectivity and PrP^{Sc} within lymphoid tissues 70 days post-inoculation.

Draining ILNs and spleen tissues were taken from two mice from each control and LTβR-Ig treatment group 70 days after inoculation with the scrapie agent by skin scarification. The scrapie infectivity titre in pooled (n = 2) tissue homogenates was estimated by bioassay in groups of indicator mice. As expected, ILNs from control mice treated with hu-Ig 3 days before or 14 or 42 days after inoculation contained high infectivity titres (6.0 - 6.8 log i.c. ID_{50}/g, as estimated by incubation period assay; Table. 5.2). After treatment of mice with LTβR-Ig 3 days before inoculation with the scrapie agent, scrapie infectivity was undetectable in ILNs suggesting a scrapie infectivity titre, if present, below 3.5 log i.c. ID_{50}/g (at least 1000 fold less than the level detected in ILNs of control-treated mice assayed at the same time post-inoculation) (Table. 5.2). Thus these data suggest that temporary dedifferentiation of FDCs before inoculation with the scrapie agent blocks the early accumulation of scrapie infectivity in the draining ILN. Analysis of ILNs from mice treated with LTβR-Ig 14 or 42 days after inoculation, suggested they contained detectable but significantly lower levels of infectivity than those measured in ILNs from control treated mice (Table. 5.2).

Table 5.2- Effect of LTβR-Ig treatment on the accumulation of scrapie infectivity in draining inguinal lymph nodes 70 days post-inoculation by skin scarification as determined by a incubation period bioassay [a].

	hu-Ig			LTβR-Ig		
Day of treatment	Incidence[b]	Mean incubation period (days) ± S.E.M	Titre[c]	Incidence[b]	Mean incubation period (days) ± S.E.M	Titre[c]
-3	8/8	192 ± 6	6.8	0/8	9X> 300	<3.5
+14	8/8	203 ± 4	6.3	9/9	226 ± 10	5.0
+42	7/7	192 ± 6	6.0	6/8	265 ± 10, 2X >300	3.8

[a]; Mice were given a single i.p. injection (100 μg) of LTβR-Ig or hu-Ig as a control on the days indicated before or after inoculation with scrapie via skin scarification of the right thigh. Inguinal lymph nodes draining the site of inoculation were pooled from two mice and infectivity levels determined by i.c. injection of lysates into groups of C57BL/Dk indicator mice.

[b]; Incidence = number of animals affected/number of animals tested. The notation "N X > 300" means that mice were free of the signs of scrapie up to at least this time after inoculation.

[c];Scrapie infectivity titres expressed as log i.c. 50% infectious dose (ID_{50})/g.

Table 5.3- Effect of LTβR-Ig treatment on the accumulation of scrapie infectivity in the spleen 70 days post-inoculation by skin scarification as determined by incubation period bioassay[a].

	hu-Ig			LTβR-Ig		
Day of treatment	Incidence[b]	Mean incubation period (days) ± S.E.M	Titre[c]	Incidence[b]	Mean incubation period (days) ± S.E.M	Titre[c]
-3	9/9	187 ± 3	7.1	0/9	9X>300	<3.5
+14	9/9	208 ± 9	6	3/8	295 ± 6 , 5X>300	<3.5
+42	8/9	187 ± 4	7	6/9	273 ± 11, 3X>300	3.7

[a] ;Mice were given a single i.p. injection (100 µg) of LTβR-Ig or hu-Ig as a control on the days indicated after inoculation with scrapie via skin scarification of the right thigh. Spleens were pooled from two mice and infectivity levels determined by i.c. injection of lysates into groups of C57BL/Dk indicator mice.

[b];Incidence = number of animals affected/number of animals tested. The notation "N X > 300" means that mice were free of the signs of scrapie up to at least this time after inoculation.

[c]; Scrapie infectivity titres expressed as log i.c. 50% infectious dose (ID_{50})/g.

Similarly, spleen tissue from each group of control-treated mice contained high levels of scrapie infectivity when measured 70 days after inoculation (6.0 – 7.1 log i.c. ID_{50}/g). However, after treatment of mice with LTβR-Ig 3 days before or 14 days after inoculation with the scrapie agent, infectivity was undetectable in the spleen suggesting a scrapie infectivity titre, if present, below 3.5 log i.c. ID_{50}/g (at least a 1000 fold less than the level detected in spleens of control-treated mice assayed at the same time post-inoculation; (Table. 5.3). However, spleen tissue from mice treated with LTβR-Ig 42 days after inoculation, contained trace levels of infectivity (3.7 log i.c. ID_{50}/g) but significantly lower levels of infectivity (at least 100 fold less than those measured in spleen tissues from control-treated mice; (Table 5.3).

The level of PrP^{Sc} accumulation was also determined in spleen tissue from both control and treated mice 70 days after inoculation. As expected, spleens from control mice contained abundant accumulations detergent insoluble proteinase K (PK) resistant PrP^{Sc} (Fig. 5.4, lanes 2 and 4). A typical three-banded pattern was observed between the molecular mass values of 20-30 kDa, representing the unglycosylated, monoglycoslated and diglycoslated isomers of PrP (in order of increasing mass). However, after treatment with LTβR-Ig, either before or shortly after inoculation with the scrapie agent, no accumulations of PrP^{Sc} were detected (Fig. 5.4, lanes 6 and 8). These data demonstrate that blockade of the LTβR signalling pathway impairs the accumulation of the scrapie agent within MLNs and spleen tissue up to 70 days post-inoculation.

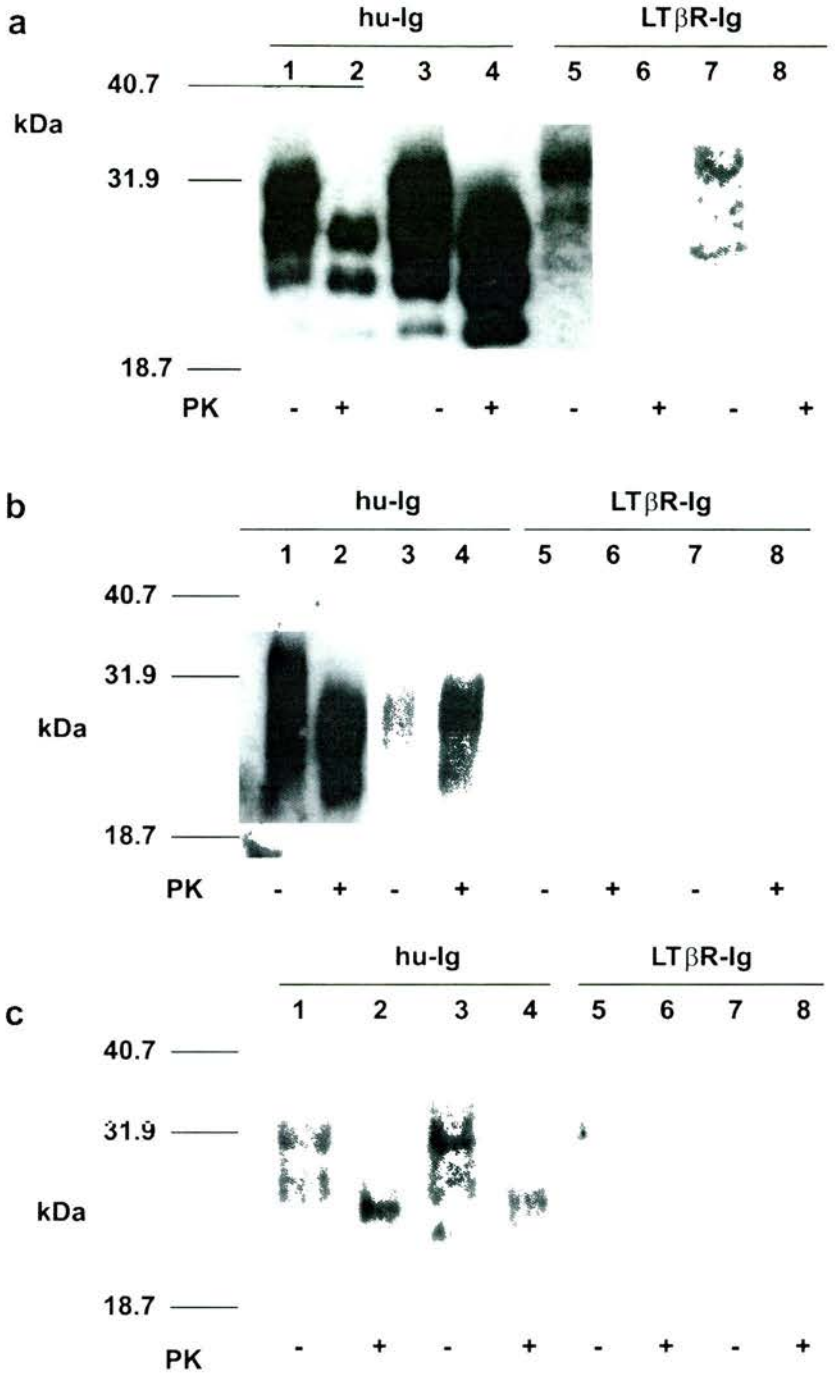


Figure 5.4– Treatment with LTβR-Ig prior to or shortly after scrapie inoculation via skin scarification blocks the early accumulation of PrP^{Sc} in spleen tissue (a, b and c) taken 70 days post-inoculation with scrapie. Mice were treated with LTβR-Ig or hu-Ig (control) either, 3 days before (a) or 14 (b) or 42 (c) days after scrapie challenge. Samples were treated in the absence (-) or presence (+) of proteinase K (PK) prior to electrophoresis. Abundant levels of PrP^{Sc} accumulation were detected in the spleens of all hu-Ig treated mice (lanes 2 and 4). No PrP^{Sc} was detected in the spleens of LTβR-Ig treated mice (lanes 6 and 8).

5.3.4 Effect of LTβR-Ig treatment on the accumulation of PrP^{Sc} in lymphoid tissues from terminally scrapie affected mice

To determine whether LTβR-Ig treatment affected the accumulation of PrP^{Sc} within lymphoid tissues of terminally scrapie affected mice, ILNs and spleen tissues were collected from all hu-Ig and LTβR-Ig treated mice that developed the clinical signs of scrapie. Immunoblot analysis of pooled ILNs and spleen tissue ($n = 2$) taken from terminally scrapie-affected control mice detected abundant accumulations of PrP^{Sc} (Fig. 5.5, Fig. 5.6). Abundant accumulations of PrP^{Sc} were also detected in the ILNs (Fig. 5.5) and spleen tissue (Fig. 5.6) of all LTβR-Ig treated mice which developed clinical signs of scrapie. The effects of LTβR-Ig treatment on FDC dedifferentiation are temporary and mature FDC networks begin to reappear approximately 28 days after treatment (Mabbott et al., 2003; Mackay and Browning, 1998). Therefore, the detection of abundant PrP^{Sc} in ILNs and the spleen of clinically scrapie-affected LTβR-Ig treated mice are consistent with accumulation of residual PrP^{Sc} on restored FDC networks within these tissues.

5.3.5 Effect of LTβ-Ig treatment on scrapie susceptibility

Mice were treated with LTβR-Ig (or hu-Ig as a control) at one of three time points either prior to or shortly after inoculation with a 1% dilute of terminally scrapie-affected mouse brain homogenate. LTβR-Ig treatment was given either; 1) three days prior to inoculation with the scrapie agent to ensure no FDC networks were present within lymphoid follicles at the time of inoculation, 2) 14 days after inoculation with the scrapie agent shortly after the onset of PrP^{Sc} accumulation in the draining ILNs (Table 5.1) or 3) 42 days after inoculation when abundant levels of

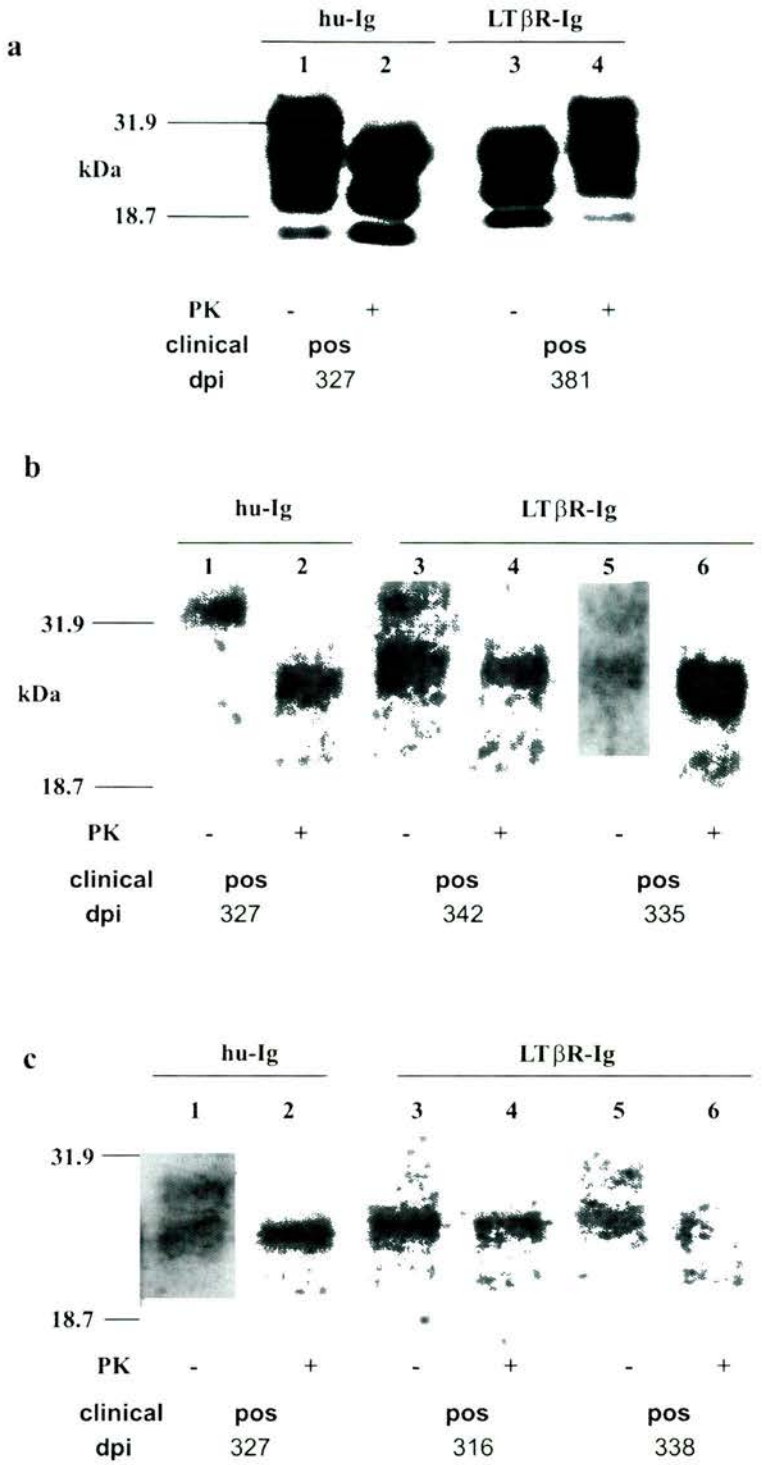


Figure 5.5- PrP^{Sc} accumulation in pooled draining inguinal lymph nodes (n = 2) from terminally scrapie-affected mice. Mice were treated with LTβR-Ig or hu-Ig (control) either, 3 days before (a) or 14 (b) or 42 (c) days after scrapie challenge. Immunoblots show the accumulation of detergent insoluble, relatively proteinase K (PK) resistant PrP^{Sc} in all treatment groups at the terminal stage of the disease. Treatment of tissues in the presence (+) or absence (-) of proteinase K (PK) prior to electrophoresis is indicated. pos., mice that developed clinical signs of scrapie; dpi, days post-inoculation at which the tissues were taken for analysis.

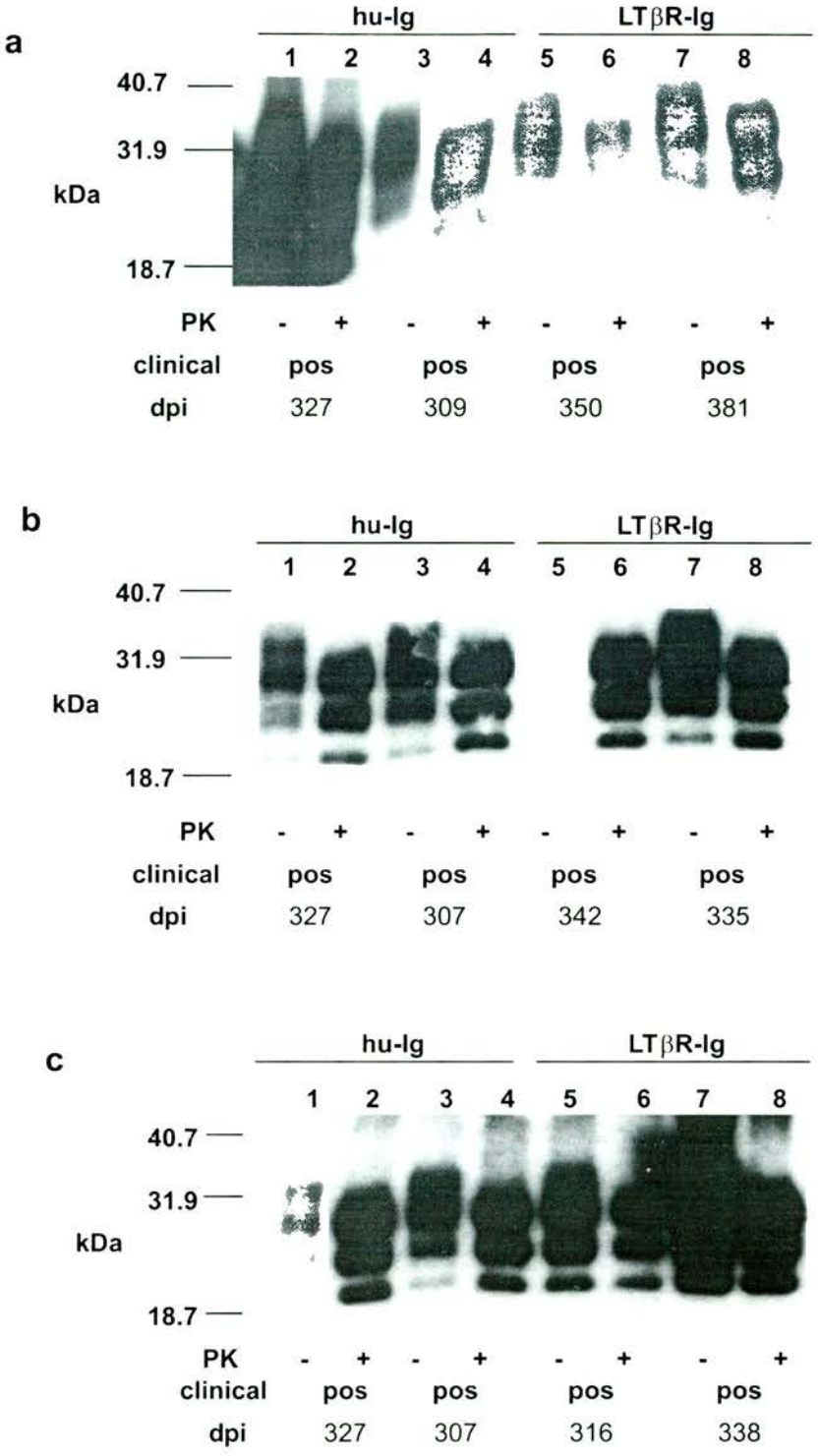


Figure 5.6- PrP^{Sc} accumulation in spleen tissue from terminally scrapie-affected mice. Mice were treated with LTβR-Ig or hu-Ig (control) either, 3 days before (a) or 14 (b) or 42 (c) days after scrapie challenge. Immunoblots show the accumulation of detergent insoluble, relatively proteinase K (PK) resistant PrP^{Sc} in all treatment groups at the terminal stage of the disease. Treatment of tissues in the presence (+) or absence (-) of proteinase K (PK) prior to electrophoresis is indicated. pos., mice that developed clinical signs of scrapie; dpi, days post-inoculation at which the tissues were taken for analysis.

PrP^{Sc} are detectable within ILNs (Table. 5.1 and Fig. 5.3).

All control mice treated 3 days before inoculation with the scrapie agent succumbed to disease with a mean incubation period of 320 ± 3 (n = 6) days (Table 5.4). In contrast three of the seven mice treated with LTβR-Ig developed disease approximately 75 days later, with individual incubation periods of 350, 381, 402 days post-inoculation (Table 5.4). These incubation periods were beyond the range observed in the -3 days hu-Ig treated control mice (309 - 327 days post-inoculation). Four of the LTβR-Ig treated mice remained free from the signs of scrapie 600 days post-inoculation (Table 5.4). These data demonstrate that treatment with LTβR-Ig 3 days prior to inoculation with the scrapie agent significantly reduces disease susceptibility and significantly extends survival time (Table 5.4).

All mice treated with LTβR-Ig 14 days after inoculation with the scrapie agent developed the neurological disease with a mean incubation period of 351 ± 4 days (n = 8), 35 days later than control treated mice ($P \leq 0.001$; Table 5.4) which had a mean disease incubation period of 316 ± 3 days (n = 8). However treatment of mice with LTβR-Ig 42 days after inoculation with the scrapie agent did not significantly affect the disease incubation period (P = 0.158) or susceptibility, when compared to control treated mice (Table 5.4). Together these data show that blockade of the LTβR signalling pathway 3 days before inoculation with the scrapie agent can significantly extend the disease incubation period and reduce disease susceptibility. Furthermore, when LTβR-Ig was given 14 days after inoculation with the scrapie agent the disease incubation period is significantly extended. The absence of an

Table 5.4- Effect of LTβR-Ig treatment on scrapie susceptibility when inoculated by skin scarification[a].

	hu-Ig		LTβR-Ig	
Day of treatment	Incidence[b]	Mean incubation period (days) ± S.E.M	Incidence	Mean incubation period (days) ± S.E.M
-3	6/6	320 ± 3	4/7	443 ± 23 3X> 557
+14	8/8	316 ± 3	8/8	351 ± 4
+42	7/7	328 ± 6	8/8	343 ± 8

[a]; Mice were given a single i.p. injection (100 µg) of LTβR-Ig or hu-Ig as a control on the days indicated before or after inoculation with scrapie via skin scarification of the right thigh.

[b]; Incidence = number of animals affected/number of animals tested. The notation "N X > 557" means that mice were free of the signs of scrapie up to at least this time after inoculation.

effect when LTβR-Ig treatment was given at 42 days after inoculation with the scrapie agent is consistent with the hypothesis that the scrapie agent has already spread to the peripheral nervous system at this time point.

Histopathological analysis of brain tissue from terminally scrapie-affected mice from each treatment or control group displayed the characteristic spongiform pathology, gliosis and disease-specific PrP accumulations typical of a peripheral inoculation with the ME7 scrapie strain (Fig. 5.7). However, no characteristic ME7 specific histopathology was observed in the brains of mice from -3 days LTβR-Ig treatment group which did not succumb to scrapie infection (Fig 5.7). No significant difference in the severity or distribution of the pathological targeting of vacuolation in the brain was observed between treatment and control groups (Fig. 5.8). These data demonstrate that neuroinvasion had occurred via a common pathway in the control and LTβR-Ig treated mice that developed clinical disease.

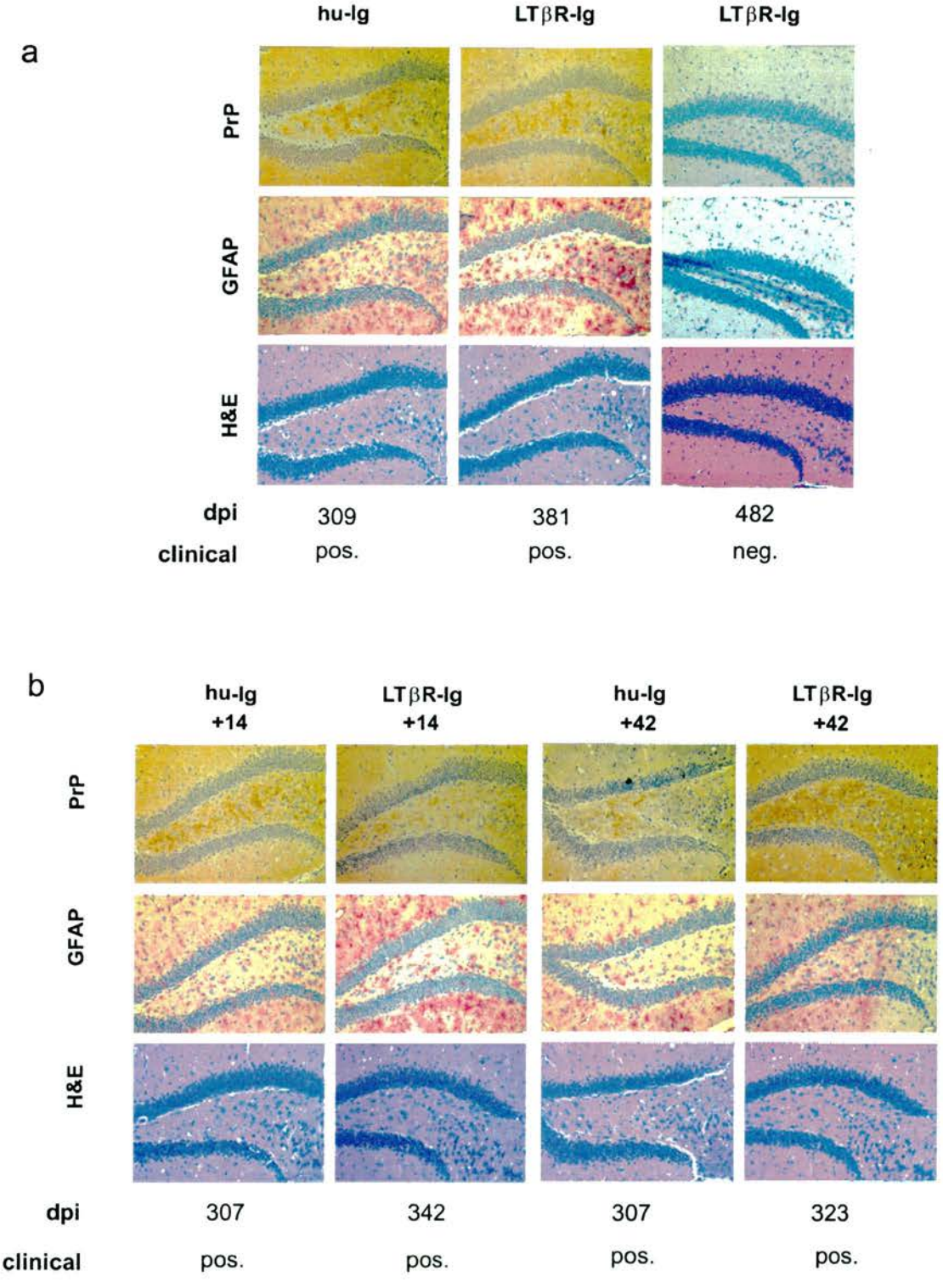


Figure 5.7- Histological analysis of brain tissue from terminally scrapie affected mice which received either LTβR-Ig or hu-Ig (control) treatment either 3 days before (a) or 14 or 42 days after (b) scrapie inoculation by skin scarification. Large PrP accumulations (brown) were detected in the hippocampi of all mice which developed clinical signs of scrapie (upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red) shows diffuse gliosis in the hippocampi of all terminally scrapie affected mice (middle row). Adjacent sections were also stained with hematoxylin and eosin and all displayed extensive vacuolation (bottom row). All sections were counterstained with hematoxylin (blue). Original magnification X200. dpi; days post-inoculation tissues were taken for analysis.

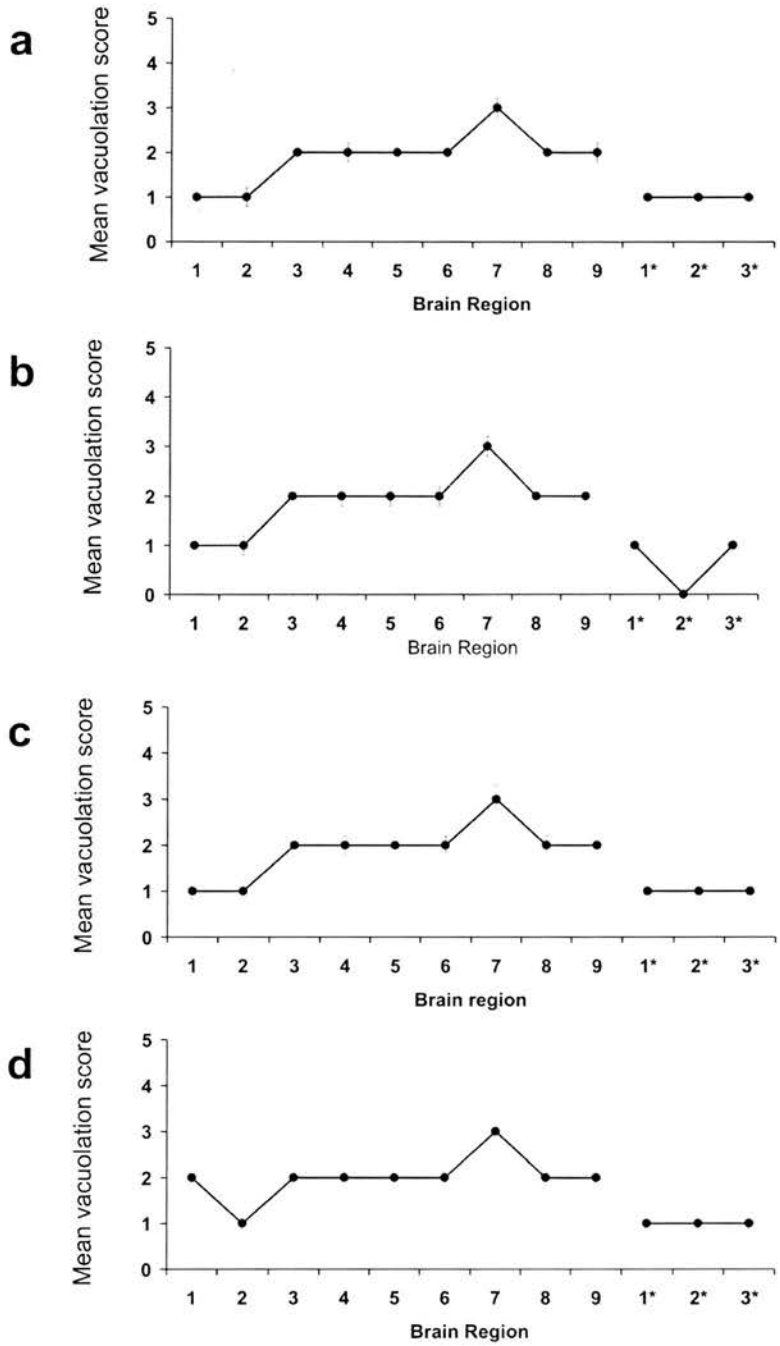


Figure 5.8- Treatment with the LTβR-Ig either 3 days before (a) or 14 (b) or 42 (c) days after inoculation with scrapie strain ME7 by skin scarification does not effect pathological targeting of vacuolation in the brain when compared to hu-Ig treated controls (d). Vacuolation in the brain was scored on a scale of 0-5 in the following grey-matter (G1-G9) and white-matter (W1-W3) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, Decoction of superior cerebellar peduncles; W3, Cerebral peduncles. Each point represents mean vacuolation score ± S.E.M for groups of 5-9 mice.

5.4 Discussion

In order to further investigate the role of FDCs in scrapie pathogenesis following inoculation via the skin, FDCs were temporarily dedifferentiated by a single i.p. injection of LTβR-Ig either, 3 days before or 14 or 42 days after inoculation with the scrapie agent by skin scarification. Data presented here shows that LTβR-Ig treatment blocked the early accumulation of scrapie infectivity and PrP^{Sc} in the draining ILNs and spleen. These effects coincide with the temporary dedifferentiation of FDCs in ILNs and the spleen. LTβR-Ig treatment 3 days before inoculation with the scrapie agent significantly extended the disease incubation period and reduced disease susceptibility, when compared to control mice. Treatment with LTβR-Ig 14 days after inoculation with the scrapie agent also significantly extended the disease incubation period but did not affect disease susceptibility. In contrast, LTβR-Ig treatment at 42 days after inoculation with the scrapie agent did not affect the disease incubation period or disease susceptibility, suggesting that infectivity may have already began to spread to the peripheral nervous system by this time point.

LTβR-Ig treatment, either before or shortly after inoculation with the scrapie agent by skin scarification, resulted in the loss of functional FDCs within draining ILNs and spleen tissue within 3 days of treatment. FDCs functionally trap and retain antigens on their surface through interactions with complement components and cellular complement receptors (Nielsen et al., 2000; Pepys, 1976). Data presented here demonstrates that LTβR-Ig treatment resulted in the temporary loss of complement receptor 1 (CD35) and complement component C4 (FDC-M2;) (Taylor

et al., 2002) specific immunostaining in the ILNs and spleen within 3 days of treatment. Therefore these data suggest that any FDCs if present in LTβR-Ig treated mice would be unable to trap and retain complement opsonized antigens. Previous research has suggested that complement and cellular complement receptors may play a role in the localization and retention of PrP^{Sc} to FDCs (Klein et al., 2001; Mabbott et al., 2001). In addition recent research has suggested that C1q may induce the up-regulation of PrP^{C} by FDCs rendering the host more susceptible to scrapie infection (Lotscher et al., 2003). Taken together these data would suggest that the capacity of FDCs to capture and retain PrP^{Sc} and scrapie infectivity through association with complement components would be abolished after LTβR-Ig treatment.

The fate of FDCs after LTβR-Ig treatment is unknown but several mechanisms could contribute to their temporary absence in lymphoid tissues: Firstly, FDCs could dedifferentiate to an immature state that lacks the ability to trap and retain antigen; secondly FDCs may undergo apoptosis, or alternatively LTβR-Ig may disrupt chemokine gradients responsible for the organisation of lymphoid follicles resulting in the dispersal of FDCs. Each of these possibilities would result in the loss of immunostaining for the FDC markers FDC-M1, FDC-M2 and CD35. Further research is necessary to determine the precise fate of FDCs after LTβR-Ig treatment.

After inoculation with the scrapie agent, control mice accumulated detectable amounts of PrP^{Sc} in their right ILNs draining the site of inoculation within 17 days after inoculation. The subsequent detection of PrP^{Sc} in the left non-draining ILN and spleen further contributes to the hypothesis (Chapter 3) that the scrapie agent

disseminates systemically to other lymphoid tissues from the right draining ILN via the bloodstream. In contrast, mice which received LTβR-Ig at 14 or 42 days after inoculation with the scrapie agent had either visibly reduced accumulations or undetectable levels PrP^{Sc} within the ILNs and spleen within 3 days of treatment. The absence or reduction of PrP^{Sc} coincides with the loss of FDCs in the ILNs and spleen. The loss of PrP^{Sc} accumulations within in these lymphoid tissues is most likely due to the release PrP^{Sc} from complement receptors expressed by FDCs, and its clearance by phagocytic cells (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982; Luhr et al., 2004; Luhr et al., 2002).

After skin scarification of immunocompetent mice with the ME7 scrapie strain, a high level of infectivity accumulate within the spleen by 63 days post-inoculation, and subsequently plateaus for the remainder of the disease (Chapter 3; Fig. 3.1). In this study, pooled ILNs and spleen tissues from all control mice, contained high levels of scrapie infectivity at 70 days post-inoculation. However, when mice were treated with LTβR-Ig 3 days before inoculation with the scrapie agent infectivity was undetectable within the ILNs at 70 days post-inoculation, approximately 40 days after the expected reappearance of mature FDCs. Similarly only trace levels of infectivity and no PrP^{Sc} were detected in the spleen of the same mice at 70 days post-inoculation.

In contrast pooled ILNs from mice treated with LTβR-Ig either 14 or 42 days post-inoculation did contain low levels of infectivity (approximately 4.4 log i.c ID_{50}/g). Similarly, trace levels of infectivity were detected in the spleen 70 days post-

inoculation in mice which were treated with LTβR-Ig 42 days post-inoculation. ILNs and spleen tissue from LTβR-Ig treated mice which developed the clinical signs of scrapie contained abundant accumulations of PrP^{Sc} in these lymphoid tissues at the terminal stage of the disease.

The most likely explanation for these observations is that in the temporary absence of FDCs, a proportion of the original inoculum is destroyed by phagocytic cells but a low level of scrapie infectivity is able to persist. Therefore upon the re-appearance of FDCs, the fraction of scrapie infectivity which had persisted would then be able to initiate replication on regenerated FDC networks, resulting in the accumulation of the scrapie agent in lymphoid tissues at the terminal stage of the disease. This hypothesis is consistent with the observation that LTβR-Ig treatment prevents the early accumulation of the scrapie agent within lymphoid tissues in the absence of FDC networks but high levels of PrP^{Sc} and infectivity are detectable at the terminal stage of the disease following the subsequent reappearance of FDC networks.

Data in this chapter demonstrates that a temporary absence of FDCs at the time of inoculation results in a significantly extended disease incubation period and reduced disease susceptibility. The reduced disease susceptibility of mice which received LTβR-Ig treatment 3 days before inoculation with the scrapie agent is most likely due to the clearance of scrapie infectivity by phagocytic cells such as macrophages (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982) or dendritic cells (Luhr et al., 2004; Luhr et al., 2002) both, of which have been shown to have the potential to sequester and degrade the scrapie agent. The extended

survival time in mice treated with LTβR-Ig 3 days before inoculation with the scrapie agent suggests that in some mice a fraction of inoculm persists until the reappearance of FDCs 28 days after treatment, allowing for replication of the scrapie agent to resume. However, the delay in onset of the clinical disease (approximately 75 days later than controls) is greater than the 28 day time period which FDCs are absent from the lymphoid tissues. This data suggests that the may be variations in the clearance of the scrapie agent from the host in the absence of FDCs resulting in variable rates of replication of scrapie infectivity following their reappearance and thus subsequently altering the onset of the clinical disease.

LTβR-Ig treatment 14 days after inoculation with the scrapie agent significantly extends the disease incubation but has no effect on disease susceptibility. Data suggests that PrP^{Sc} had already begun to accumulate on FDCs within the lymphoid tissues prior to LTβR-Ig treatment at 14 days post-inoculation. Thus, the extended disease incubation period in the absence of reduced disease susceptibility is most likely due to the accumulation of infectivity prior to LTβR-Ig treatment, which may have been at a level that was sufficient to avoid substantial clearance by phagocytic cells in the absence of FDCs. Residual scrapie infectivity would be unable to replicate until the reappearance of mature FDCs 28 days after treatment, delaying subsequent neuroinvasion and resulting in a protracted disease incubation period.

Treatment with LTβR-Ig has no affect on disease pathogenesis once infection is established within the peripheral or central nervous system (Mabbott et al., 2000a; Mabbott et al., 2003). The lack of any observable effect of LTβR-Ig treatment on

disease pathogenesis when given 42 days after inoculation with the scrapie agent is consistent with the hypothesis that neuroinvasion had already occurred. It also suggests that early neuroinvasion may have occurred directly from the draining ILN which had a heavy deposition of PrP^{Sc} prior to LTβR-Ig treatment at 42 days post-inoculation, whereas the spleen had accumulated only limited amounts of PrP^{Sc} in some mice at this time point. Removal of the spleen prior to i.p. inoculation with the scrapie agent significantly extends the disease incubation period, suggesting an important role for this tissue in neuroinvasion after i.p. inoculation (Fraser and Dickinson, 1970). In contrast, removal of the spleen prior to subcutaneous inoculation with the scrapie agent had no effect on the disease incubation period or disease susceptibility (Fraser et al., 1992). Taken together these data suggest, as discussed previously in Chapter 3, that the spleen is unlikely to play a critical role in peripheral scrapie pathogenesis following inoculation via the skin.

Treatment with LTβR-Ig can inhibit or prevent the development of experimental autoimmune encephalomyelitis (Gommerman et al., 2003). In this model the LTβR-mediated disease prevention is associated with defects in T-lymphocyte responses and migration (Gommerman et al., 2003). LIGHT is a 29 kDa type II transmembrane protein produced by activated T-lymphocytes that also binds to LTβR (Mauri et al., 1998). However, the effects of LTβR-Ig treatment on scrapie pathogenesis are unlikely to be due to impaired LTβR or LIGHT-mediated T-lymphocyte responses as previous studies have shown that pathogenesis is unaffected in mice deficient in T-lymphocytes (Fraser and Dickinson, 1978; Klein et al., 1997; Klein et al., 1998). Signalling via LTβR has been shown to be important for the

presence of migratory dendritic cells in the spleen (Wu et al., 1999). Therefore, it is plausible that blockade of the LTβR-signalling pathway might have affected cell trafficking or the transport of scrapie infectivity to the draining ILN. However, data presented here shows that treatment with LTβR-Ig 14 days after inoculation with the scrapie agent significantly extends the survival time. These data suggest it is unlikely that the effects of treatment on scrapie pathogenesis are due to affects on cell trafficking as dendritic cells migrate to draining lymphoid tissues within hours of antigen encounter (Banchereau et al., 2000). Collectively these observations suggest it is highly unlikely that the major affects of LTβR-Ig-treatment on scrapie pathogenesis are independent of its effects on FDC maturation.

Previous studies by Mabbott *et al* (Mabbott et al., 2000a; Mabbott et al., 2003) and others (Montrasio et al., 2000) have shown that treatment with LTβR-Ig before either i.p. or oral inoculation with the scrapie agent significantly reduces disease susceptibility and extends the survival time. However, LTβR-Ig treatment after inoculation with the scrapie agent has varying affects on disease pathogenesis. The affect of LTβR-Ig treatment on disease pathogenesis is lost once infectivity reaches the peripheral or central nervous system. Following oral challenge LTβR-Ig treatment is ineffective 14 days after challenge, suggesting that infectivity progresses quickly from gut associated lymphoid tissues to the peripheral nerves (Mabbott et al., 2003). In contrast, following i.p. inoculation LTβR-Ig treatment is effective up to 42 days after inoculation (Mabbott et al., 2000a). Data presented in this chapter demonstrate that LTβR-Ig treatment is effective up to 14 days after inoculation but ineffective 42 days after inoculation. These data suggest that the time period in

which FDCs can be manipulated to alter disease pathogenesis varies considerably depending on the route of exposure.

Data presented here shows that mature FDCs are critical to the replication and subsequent neuroinvasion of the scrapie agent after inoculation via skin scarification. In addition these data show that the scrapie agent first accumulates in the draining ILN prior to spreading to other lymph nodes and the spleen. These data also suggest that neuroinvasion is most likely to occur between 17 – 45 days post-inoculation. Furthermore, the spleen is unlikely to play a critical role in scrapie pathogenesis after inoculation via the skin as the treatment is ineffective at a time when only low levels of PrP^{Sc} are detectable in the spleen. LTβR-Ig treatment offers a potential for therapeutic intervention after TSE exposure via the skin. However, this treatment is only effective when administrated in the short period prior to progression of the scrapie agent to the peripheral nerves.

6

Langerhans cells acquire and degrade PrP^{Sc} in vitro

6.1 Abstract

Langerhans cells (LCs) are specialized antigen presenting cells that continually sample their microenvironment within the epidermis and are able to acquire both self and non-self molecules. The ability of LCs to acquire and capture antigens suggests that this cell type would be likely to interact with the scrapie agent after inoculation via the skin. XS106 cells are a mature LCs-like cell line isolated from mouse epidermis. To investigate the potential interaction of LCs with the scrapie agent, XS106 cells were exposed to scrapie-affected brain homogenate *in vitro*. Data presented here demonstrates that XS106 cells rapidly acquire PrP^{Sc} following *in vitro* exposure. In addition, XS106 cells appear to partially degrade PrP^{Sc} and scrapie infectivity over a 96-hour observation period. These data suggest that LCs might acquire and degrade the scrapie agent after inoculation via the skin. Interestingly, the ability of XS106 cells to degrade PrP^{Sc} is lost following pre-treatment with LPS. This suggests the potential of LCs to degrade the scrapie agent might be lost in the presence of antigens such as LPS or other immunostimulatory molecules. Exposure of XS106 cells to the scrapie agent did not alter their surface expression of PrP^{C} or the following co-stimulatory or activation markers: CD40, CD80, CD86, Ia^{k}, CD205, CD54 and CD11c. These data suggest that LCs would not undergo maturation following inoculation with the scrapie agent via the skin as the expression of surface markers associated with LC maturation were unaltered *in vitro*.

6.2 Introduction

Following inoculation via the skin the scrapie agent first accumulates within the draining lymph node (DLN) (Chapter 3 and Chapter 5). However it is not known how the scrapie agent is transported from the site of inoculation to the DLN. Langerhans cells (LCs) are specialised antigen presenting cells (Banchereau and Steinman, 1998). They continually sample their microenvironment and are able to acquire both self and non-self molecules by a number of mechanisms such as; phagocytosis, macropinocytosis or by mediated adsorptive endocytosis (Garret and Mellman, 1999). Upon antigen encounter LCs receive a complex network of signals which stimulate their maturation and migration to draining lymphoid tissue where they present antigen in a MHC-class restricted manner to T-lymphocytes, initiating a primary immune response (Kimber et al., 1999). The ability of LCs to recognise and acquire antigen in the epidermis and then transport it to the DLN suggested that this cell type is a likely candidate for the uptake and transport of the scrapie agent after inoculation via the skin. Furthermore, previous research has shown that a small sub-population of dendritic cells are capable of transporting PrP^{Sc} from the gut lumen to the mesenteric lymph nodes (Huang et al., 2002).

XS106 cells are a long-term, mature, LC-like cell line isolated from the epidermis of A/J mice (Timares et al., 1998) and have a strong similarity to LCs *in vivo*. These cells have been previously characterised as being LC-like by their surface phenotype (MHC II - Ia^{high}, $CD80^{high}$ and $CD86^{high}$), growth factor requirements (GM-CSF) and their potent capacity to activate naïve T-lymphocytes in an allogeneic mixed lymphocyte reaction (Timares et al., 1998; Xu et al., 1995a; Xu et al., 1995b).

The XS106 cell line has been previously used as a tool to study the potential of genetically modified dendritic cells for use in vaccine strategies (Hayashi et al., 2000; Matsue et al., 1999; Timares et al., 1998), and to study the expression and function of antigens by LCs cells (Kodali et al., 2003; Mizumoto et al., 2002). These cells have also been used to study dendritic cell responses to lipopolysaccaharide (LPS) and the hepatitis C virus (Lu et al., 2003; Sarobe et al., 2003). In this chapter XS106 cell line has been used to study the potential interaction of LCs with the scrapie agent.

Experiments performed in this chapter were designed to answer the following questions:

1) After exposure to the scrapie agent *in vitro* are LCs able to recognise and associate with the scrapie agent?
2) Do LCs degrade or replicate the scrapie agent?
3) Does the surface expression of co-stimulatory and activation antigens on LCs change after exposure to the scrapie agent?

6.3 Results

6.3.1 Characterisation of the XS106 cell line

The XS106 and XS52 cell lines were kind gifts from Akira Takashima (Dept. Dermatology, University of Texas, U.S.A). Upon receipt of these cell lines, cells were expanded and passaged as stated in Chapter 2 (section 2.6.1) for 3 weeks to obtain a large batch culture. Cells were then aliquoted and cryo-preserved for use in subsequent experiments. For each individual experiment a new aliquot of cells was revived and cultured for 10 days prior to use. In each instance, cells were cultured and passaged in exactly the same manner to ensure homogeneity between cell populations in individual experiments.

Firstly, XS106 cells were characterised according to their cellular morphology and surface phenotype. Upon culture, XS106 cells first appear as round cells extending numerous short pseudopodia ((Xu et al., 1995a): Fig.6.1). As cell monolayers become confluent many cells become round in shape and some were released spontaneously into the culture medium. This cellular morphology is characteristic of other dendritic cell lines previously isolated from the mouse epidermis (Xu et al., 1995a).

After 10 days of *in vitro* culture the surface phenotype of XS106 cells was determined by fluorescence activated cells sorter (FACS) analysis. The 10 day time point was chosen because the cell yield was sufficient to provide enough cells for use in experiments. All antibodies were titrated prior to use to determine the concentration which would give 100% saturation. The appropriate IgG2a isotype

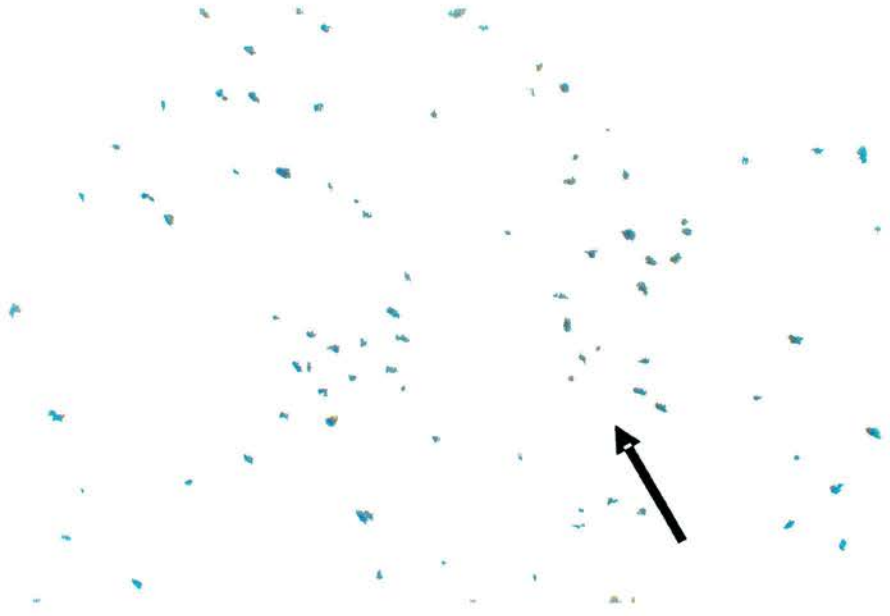


Figure 6.1- Morphology of XS106 cells after 2 hours *in vitro* culture. Cells are typically round and extend short pseudopodia (black arrow). As cultures become confluent focally cells become round and some are released spontaneously into the culture medium (white arrow). Magnification X400.

control antibodies were used in all experiments to determine background levels of non-specific binding, which was less than 1% for all samples analysed (Fig. 6.2 a-i). Analysis of the XS106 cell line after 10 days in culture demonstrated the surface phenotype of this cell line to be; $CD40^+$, $CD80^+$, $CD86^+$, Ia^{k+}(MHC-II), $CD205^+$, $CD54^+$ and $CD11c^+$(Fig. 6.2 a-i). The percentage of cells staining positive for the above surface antigens was always approximately 100%. Daily analysis of XS106 cells from days 10 to 14 in culture for the surface antigens CD40, CD80, CD86 and Ia^k demonstrated that this surface phenotype did not significantly alter as the level of expression (mean fluorescent intensity) remained unaltered (Table 6.1).

The XS106 cell line has been previously characterised as having a "mature" LC phenotype in comparison to other XS cell lines derived from the mouse epidermis due to its high expression of Ia^k, CD80 and CD86 (Timares et al., 1998; Xu et al., 1995a; Xu et al., 1995b) . Similarly in this study the mean fluorescent intensity of Ia^k, CD80 and CD86 (Fig. 6.2 b-d) by the XS 106 cell line was visibly higher than that displayed by the XS 52 cell line (Fig 6.6), and therefore the XS 106 cells were considered to be displaying a mature phenotype.

6.3.2 XS106 cells express PrP^C

Next, the expression of mRNA and protein for PrP^C by XS106 cells was determined by the reverse transcriptase polymerase chain reaction (RT-PCR) and FACS analysis respectively. Using two different sets of primers (Chapter 2; section 2.7.2) the presence of PrP^C m-RNA was confirmed by the visualisation of a single band at 1000 bp using primer combination 1 (Fig. 6.3 lanes 1 and 2) and also a single band at 400

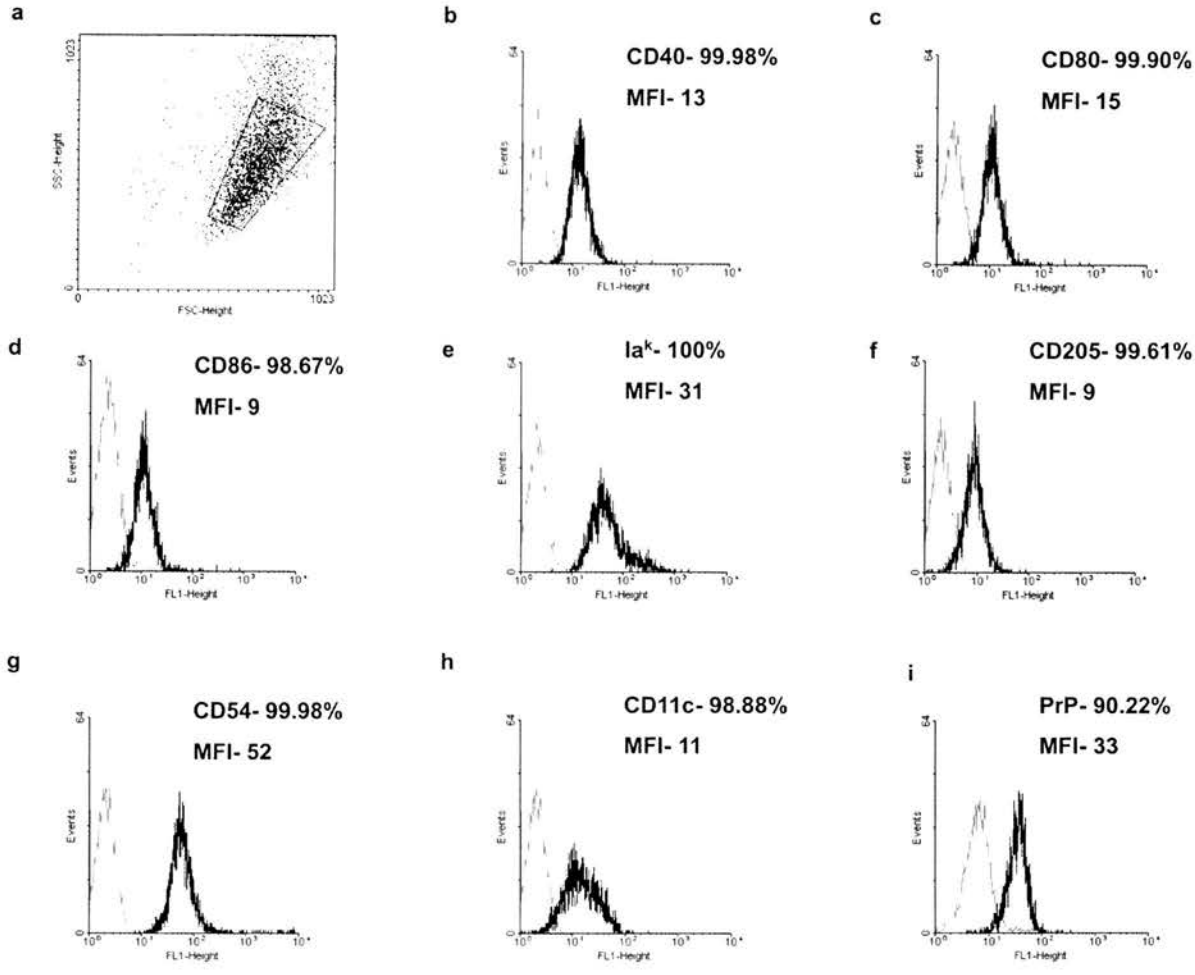


Figure 6.2- FACS analysis of the surface phenotype of XS106 cells. XS106 cells were gated as shown (a). Cells were stained with a panel of monoclonal antibodies specific for; CD40 (b), CD80 (c), CD86 (d), Ia^k (e), CD205 (f), CD54 (g), CD11c (h) and PrP (8H4; i) (black histograms) or isotype-matched controls (grey histograms) and analysed by FACS. %; percentage of cells staining positive for each surface marker. MFI; mean fluorescent intensity of cells which stained positive for the surface marker.

Table 6.1- Effect of extended cultivation on surface antigen expression by XS106 cells. XS106 cells were analysed by flow cytometry from day 10 to 14 in culture for the activation markers CD40, CD80, CD86 and Ia^k. Data represents the mean fluorescent intensity (MFI) ± standard deviation (SD).

	Days in culture				
Surface Antigen	**10**	**11**	**12**	**13**	**14**
CD40	16 ± 0	11 ± 1	13 ± 4	13 ± 4	11 ± 1
CD80	11 ± 0	12 ± 1	10 ± 0	11 ± 1	10 ± 0
CD86	12 ± 1	11 ± 1	12 ± 2	13 ± 2	12 ± 1
Ia^k	22 ± 1	18 ± 1	20 ± 1	16 ± 4	16 ± 2

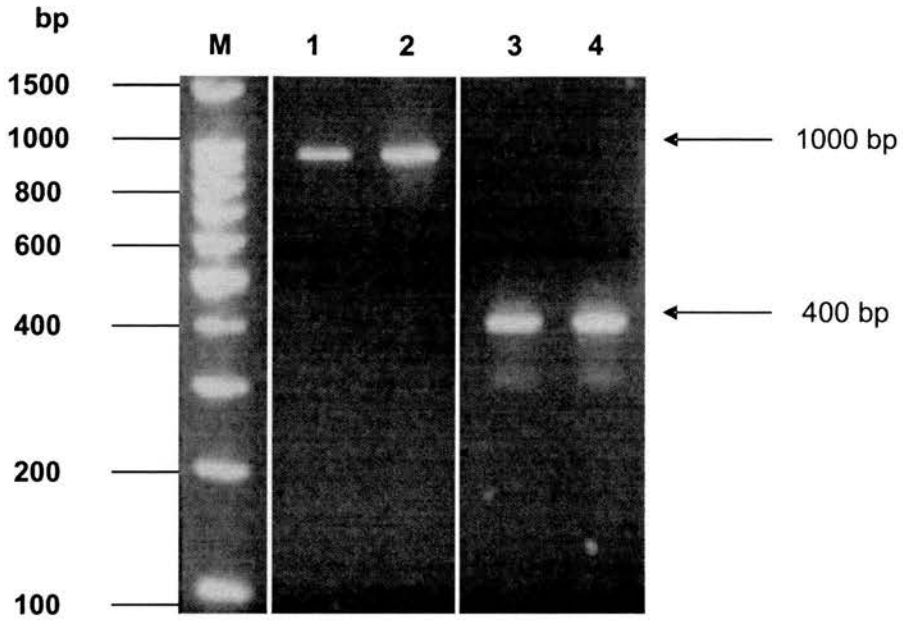


Figure 6.3- XS106 cells express mRNA for PrP^C. Expression was determined by RT-PCR analysis using two separate sets of primers. All products were resolved through gel electrophoresis containing ethidium bromide. RT-PCR analysis confirmed the presence of PrP^C m-RNA for by the visualisation of a single band at 1000 bp (lanes 1and 2) and also a single band at 400 bp (lanes 3 and 4). Lane M, 100 bp molecular marker.

bp using primer combination 2 (Fig. 6.3 lanes 3 and 4). To confirm that PrP^C was also expressed by XS106 cells at the protein level, FACS analysis was performed using the monoclonal PrP-specific antibody 8H4 (Appendix 1). The majority of cells stained positive for PrP^C (Fig. 6.2 i). Thus, these data demonstrate that XS106 cells express both PrP^C mRNA and protein.

6.3.3 XS106 cells rapidly acquire scrapie-affected brain homogenate

To determine whether XS106 cells acquire scrapie-affected brain homogenate, cells were exposed to terminally scrapie-affected brain homogenate as detailed in Chapter 2 (section 2.8.2) for either, 10 mins, 30 mins, 16 hrs or 24 hrs. Uninfected cells and cells exposed to an equivalent amount of normal brain homogenate were included as controls. Immunofluorescent confocal microscopy of cytospin preparations showed abundant accumulations of PrP in association with XS106 cells within 10 mins of exposure to scrapie-affected brain homogenate (Fig. 6.4 panel c). Visually, the strongest labelling for PrP was detected after 30 mins of exposure to scrapie brain homogenate (Fig. 6.4 panel f). Thereafter, the intensity of PrP staining appeared to decrease during the 24 hr observation period (Fig. 6.4 panels i and l). Accumulations of PrP were also detected in association with XS106 cells exposed to normal brain homogenate over the same time period (Fig. 6.4 panels, b, e, h, and k). Unfortunately, the PrP-specific antibody 8H4 does not distinguish between PrP^C and PrP^{Sc} and therefore it was not possible to determine differences between immunolabelling detected on cells exposed to normal brain to that on cells exposed to scrapie brain homogenate. In contrast, the level of PrP immunolabelling detected in association with uninfected cells was visibly less when compared to cells exposed

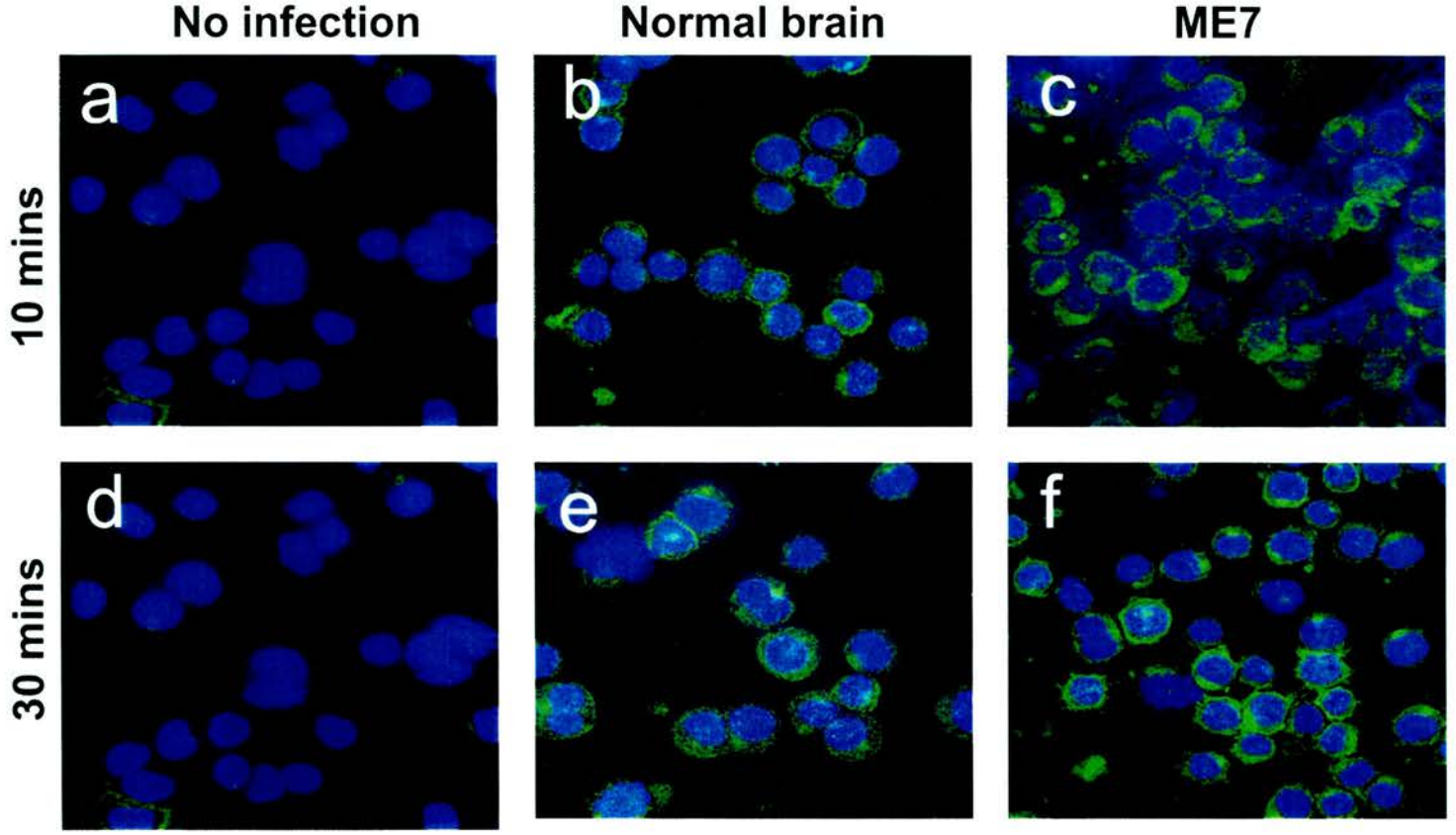


Figure 6.4 (part 1) - XS106 cells rapidly acquire scrapie brain homogenate. Immuno-fluorescent confocal analysis of XS106 cells using the PrP-specific monoclonal antibody 8H4 detected the presence of abundant intra-cellular accumulations of PrP (green) in XS106 cells exposed to scrapie brain homogenate (ME7; panels c, f, i and l) and XS106 cells exposed to normal brain homogenate (panels b, e, h and k). Cells were exposed for, 10 mins (top row), 30 mins (second row), 16 hrs (third row) or 24 hrs (bottom row). PrP was also detected in association with uninfected XS106 cells (panels a, d, g and j). Original magnification, X40. All sections were counterstained with Dapi to detect the cell nuclei (blue).

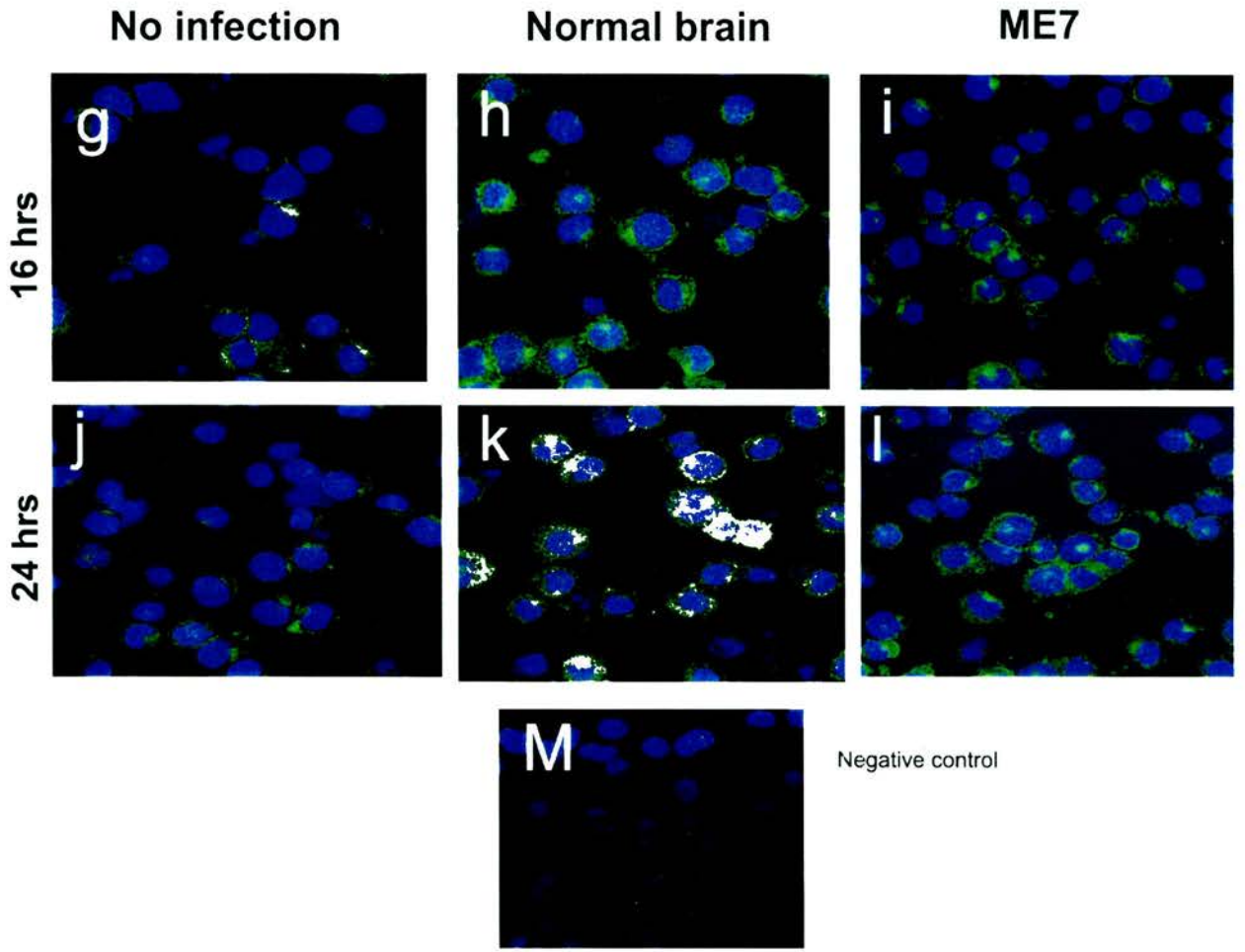


Figure 6.4 (part 2) - XS106 cells rapidly acquire scrapie brain homogenate. Immuno-fluorescent confocal analysis of XS106 cells using the PrP-specific monoclonal antibody 8H4 detected the presence of abundant intra-cellular accumulations of PrP (green) in XS106 cells exposed to scrapie brain homogenate (ME7; panels c, f, i and l) and XS106 cells exposed to normal brain homogenate (panels b, e, h and k). Cells were exposed for, 10 mins (top row), 30 mins (second row), 16 hrs (third row) or 24 hrs (bottom row). PrP was also detected in association with uninfected XS106 cells (panels a, d, g and j). Original magnification, X40. All sections were counterstained with Dapi to detect the cell nuclei (blue).

to either scrapie brain homogenate or normal brain homogenates (Fig. 6.4 panels a, d, g and j). Staining for PrP appeared to increase slightly in association with uninfected cells over the 24 hr time period studied but was visibly less than that observed in association with cells exposed to either scrapie or normal brain homogenate. Taken together, these data show that XS106 cells rapidly acquire scrapie brain homogenate after *in vitro* exposure.

6.3.4 XS106 cells degrade PrP^{Sc}

Experiments above (section 6.3.3) have shown that XS106 cells are able to acquire scrapie brain homogenate following *in vitro* exposure. However in those experiments it was not possible to determine whether the PrP detected was PrP^{Sc} or increased expression of PrP^{C} by XS106 cells. To further investigate whether XS106 cells acquire PrP^{Sc} *in vitro* cell immunoblotting experiments were performed (Chapter 2; section 2.8.4). Monolayers of XS106 cells were cultured on sterile glass coverslips and then exposed to terminally scrapie-affected brain homogenate for 16 hrs. Cells were then washed to remove excess brain homogenate and maintained in culture for between 24 hrs to 96 hrs as described in Chapter 2 (section 2.8.2). Uninfected cells and cells exposed to normal brain homogenate for the same time period were included as controls. Following inoculation, coverslips were removed and PrP^{Sc} levels determined by cell immunoblotting (Chapter 2, section 2.8.4).

XS106 cells exposed to scrapie brain homogenate and assayed immediately after washing at 16 hrs contained abundant levels of proteinase K (PK) resistant PrP^{Sc} (Fig. 6.5 a). Similarly, abundant levels of PrP^{Sc} were also detected 24 hrs post-

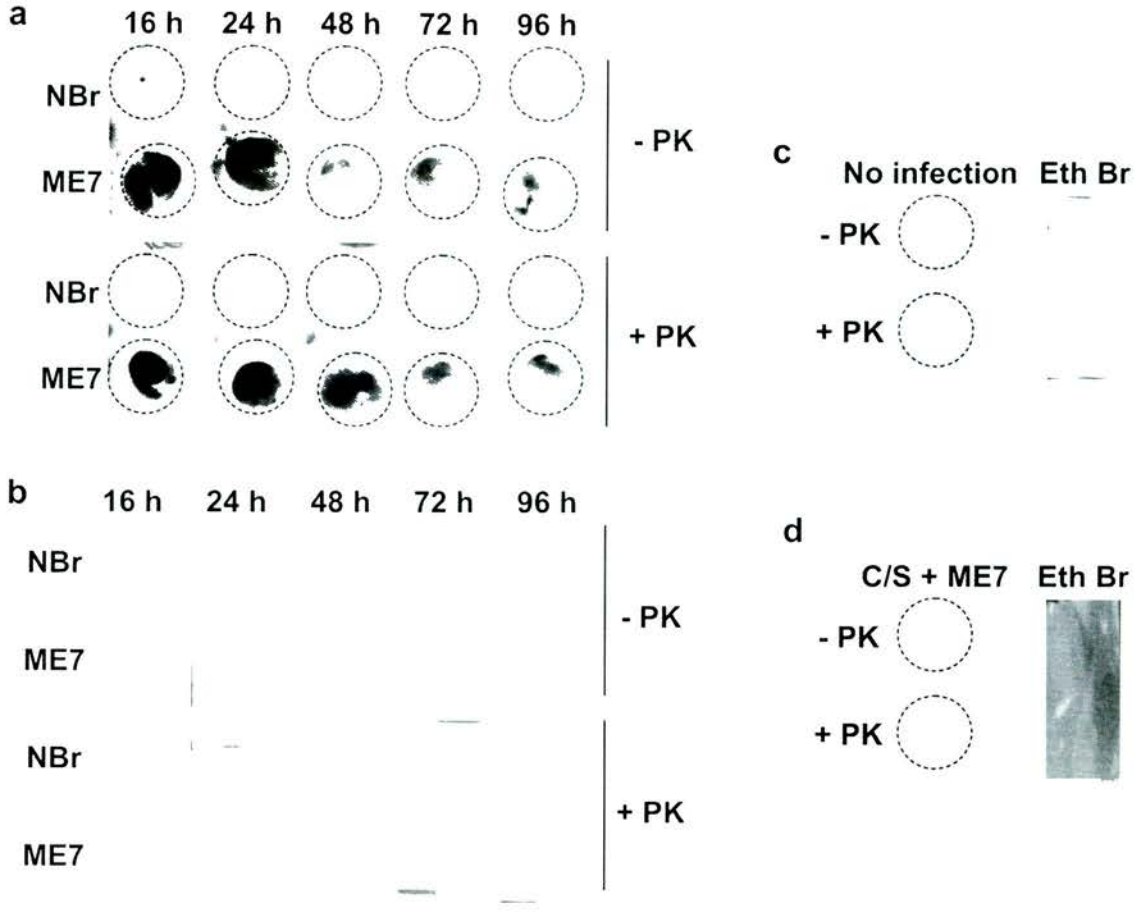


Figure 6.5- XS106 cells degrade PrP^{Sc} *in vitro* (a). Duplicate cultures of XS106 cells were exposed to either normal brain homogenate (NBr) or scrapie brain homogenate (ME7) for 16 hrs. Cells were maintained in culture for the times indicated and then transferred to PVDF membranes and membranes treated in the absence (-) or presence (+) of proteinase K (PK). Membranes were probed with the PrP-specific monoclonal antibody 8H4. Cell blot analysis showed a progressive decrease in PK resistant PrP^{Sc} in association with XS106 cells over the 96 hr observation period (a). No PrP^{Sc} was detected in association with XS106 cells exposed to normal brain homogenate (a). Nor was any PrP^{Sc} detected in association with non-infected XS106 cells (c) or coverslips exposed to scrapie brain homogenate (d). Ethidium bromide (Eth Br) staining of membranes confirmed the transfer of cellular DNA (b and c). This figure is representative of 5 separate studies.

exposure (Fig. 6.5 a). However, analysis of cells from 48 hrs onwards suggested that the levels of PrP^{Sc} detected in association with these cells progressively decreased over the 96 hr observation period (Fig. 6.5 a). In contrast, no PrP^{Sc} was detected in association with uninfected cells (Fig. 6.5 c) or cells exposed to normal brain homogenate (Fig. 6.5 a) as controls. The successful transfer of cells to the PVDF membranes was confirmed by staining the membrane with ethidium bromide to detect cellular DNA. In each instance cellular DNA was detected on membranes for each cell immunoblotblot (Fig. 6.5 b). As the XS106 cells were grown on glass coverslips further control experiments were performed to ensure that the PrP^{Sc} detected (Fig. 6.5 a) was cell associated. No PrP^{Sc} was detected in association with cell-free cover-slips exposed to scrapie brain homogenate as a control (Fig. 6.5 d), suggesting that the PrP^{Sc} detected was cell-associated and not a result of adherence to the coverslips.

6.3.5 Immature LC-like cells do not degrade PrP^{Sc} *in vitro*

Unlike the XS106 cell line used above, the XS52 cell line is an immature LCs-like cell line isolated from mouse epidermis (Xu et al., 1995a). FACS analysis of XS52 cells in this study, demonstrated that these cells do not express the surface antigens associated with LC maturation (CD40, CD80, CD86, Ia^d), suggesting that these cells were of an immature phenotype in comparison to XS106 cells (Fig. 6.6). However, these cells do express PrP^C protein (Fig. 6.6 f). The XS52 cell line was used in parallel studies to determine whether the degradation of PrP^{Sc} by XS106 cells is typical of any *in vitro* cultivated cell line exposed to the scrapie agent. Under the same experimental conditions, XS52 cells accumulated abundant PrP^{Sc} after 16 hrs

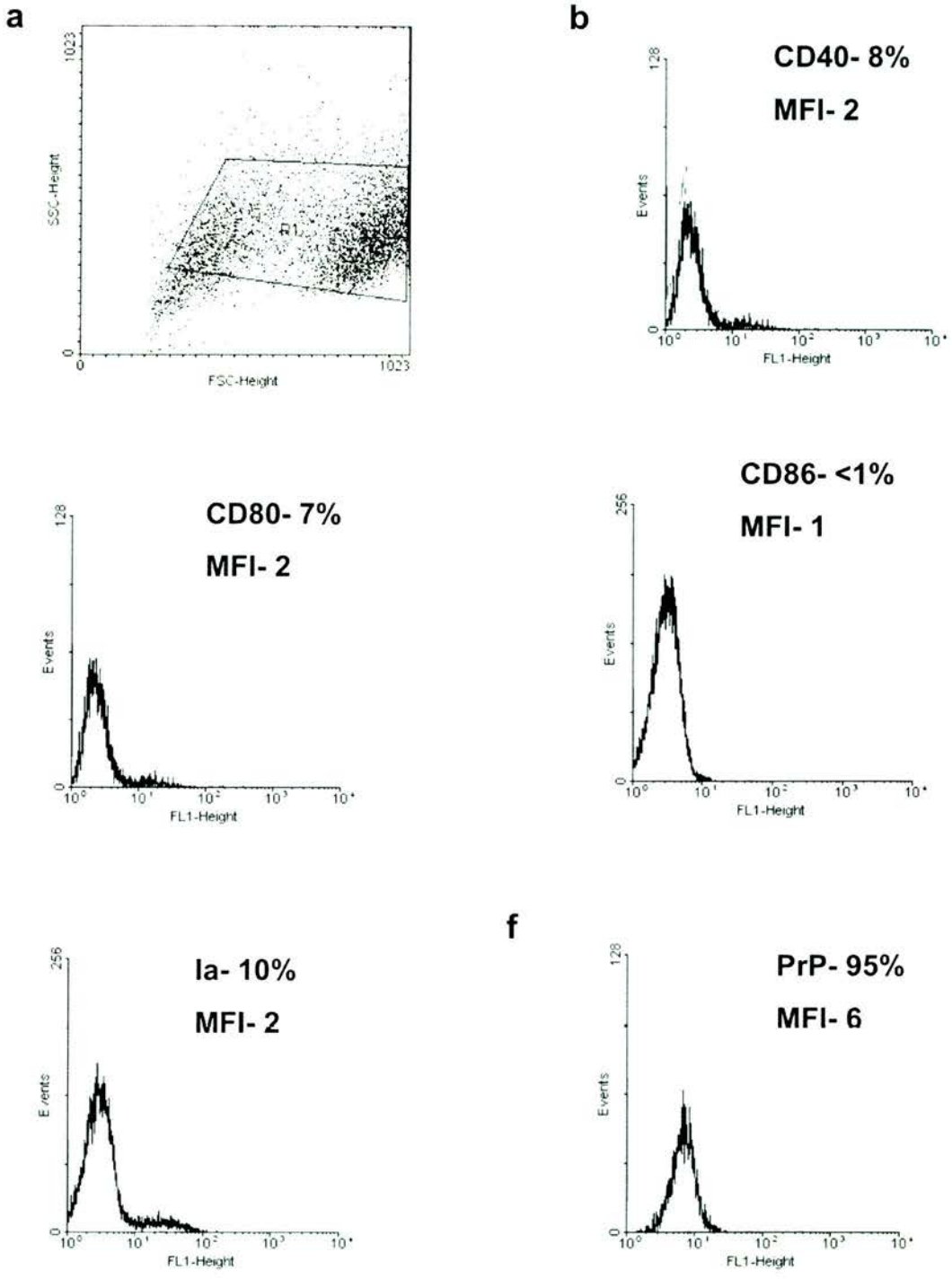


Figure 6.6- FACS analysis of the surface phenotype of XS52 cells. XS52 cells were gated as shown (a). Cells were stained with a panel of monoclonal antibodies specific for; CD40 (b), CD80 (c), CD86 (d), Ia^k (e), and 8H4 (PrP; f) (black histograms) or isotype-matched controls (grey histograms) and analysed by FACS. %; percentage of cells staining positive for each surface marker. MFI; mean fluorescent intensity of cells which stained positive for the surface marker.

exposure to scrapie brain homogenate (Fig. 6.7 a). However, in contrast to XS106 cells exposed to the scrapie agent, there was no progressive decrease in PrP^{Sc} over the 96 hr observation period (Fig. 6.7 a). Ethidium bromide staining confirmed that cells had been transferred effectively (Fig. 6.7 b). These data show that the progressive decrease of PrP^{Sc} observed in cultures of XS106 cells exposed to scrapie brain homogenate is not a property of all *in vitro* cell cultures.

6.3.6 The reduced detection of PrP^{Sc} is not due epitope masking

To confirm that the reduced detection of PrP^{Sc} in association with XS106 cells was not due to the masking the epitope recognised by monoclonal antibody 8H4 (Appendix 1) the experiment was repeated using a different PrP-specific monoclonal antibody, 7A12 (Appendix 1). The monoclonal antibody 7A12 recognises an epitope contained within amino acids 122 – 143 of the PrP protein (Zanusso et al., 1998). This epitope is distinct from the epitope recognised by 8H4 antibody which is contained within amino acids 147-164 (Zanusso et al., 1998). Experiments demonstrated that the levels of PrP^{Sc} detected following exposure of XS106 cells to scrapie brain homogenate was likewise visibly reduced over the 96 hrs observation period when the monoclonal antibody 7A12 was used to detect PrP^{Sc} (Fig. 6.8 a). Staining of membranes with ethidium bromide confirmed that cell transfer to the membranes had been successful (Fig. 6.8 b). Therefore, these data demonstrate that the reduced detection of PrP^{Sc} is not due to the masking of the epitope recognised by 8H4 antibody. Furthermore, these data confirm that XS106 cells are capable of acquiring and partially degrading PrP^{Sc}.

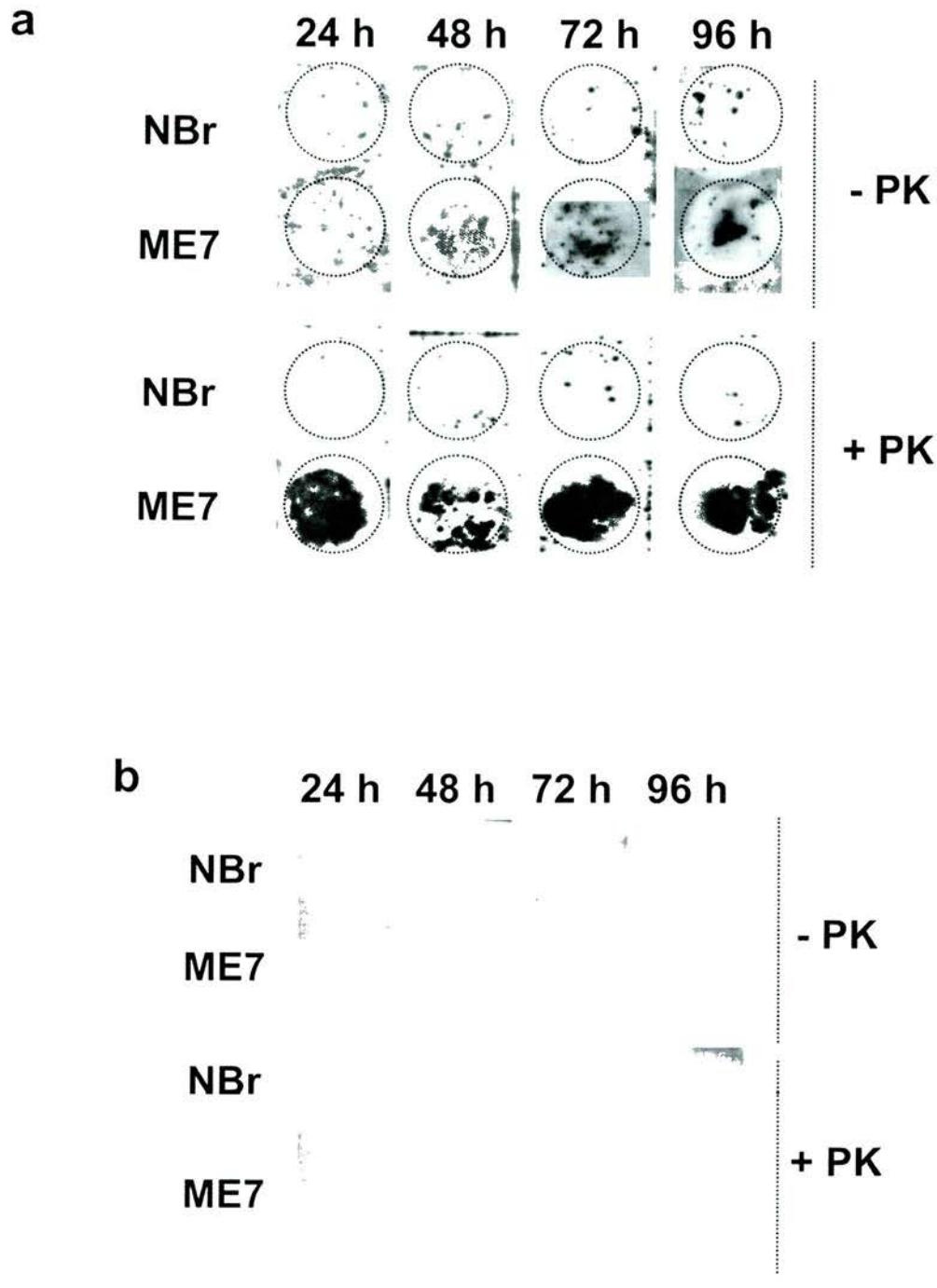


Figure 6.7- XS52 cells retain PrP^{Sc} (a). Duplicate cultures of XS52 cells were exposed to either normal brain homogenate (NBr) or scrapie brain homogenate (ME7) for 16 hrs. Cells were maintained in culture for the times indicated and then transferred to PVDF membranes and membranes treated in the absence (-) or presence (+) of proteinase K (PK). Membranes were probed with the PrP-specific monoclonal antibody 8H4. Cell blot analysis showed abundant accumulations of PrP^{Sc} in association with XS52 cells over the 96 hr observation period (a). No PrP^{Sc} was detected in association with XS52 cells exposed to normal brain homogenate (a). Ethidium bromide staining of membranes confirmed the transfer of cellular DNA (b). This figure is representative of 5 separate studies.

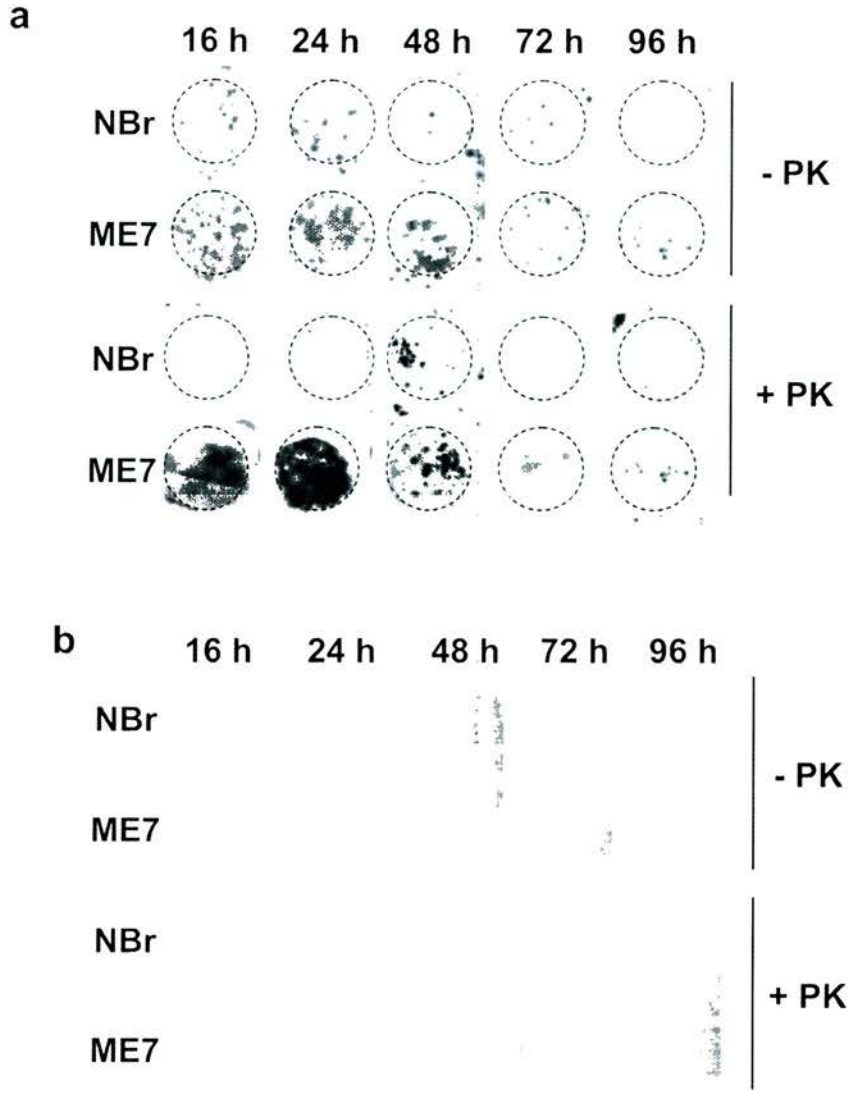


Figure 6.8- The degradation of PrP^{Sc} by XS106 cells is not due to the masking of the epitope recognised by the monoclonal antibody 8H4 (a). Duplicate cultures of XS106 cells were exposed to either normal brain homogenate (NBr) or scrapie brain homogenate (ME7) for 16 hrs. Cells were maintained in culture for the times indicated and then transferred to PVDF membranes and membranes treated in the absence (-) or presence (+) of proteinase K (PK). Membranes were probed with the PrP-specific monoclonal antibody 7A12. Cell blot analysis showed a progressive decrease PK resistant PrP^{Sc} in association with XS106 cells over the 96 hr observation period (a). No PrP^{Sc} was detected in association with XS106 cells exposed to normal brain homogenate (a). Ethidium bromide staining of membranes confirmed the transfer of cellular DNA (b). This figure is representative of two separate studies.

6.3.7 Exposure to the scrapie agent does not adversely affect the viability or metabolic activity of XS106 cells

Following exposure to scrapie brain homogenate as described in Chapter 2 (section 2.8.2) the viability of XS106 cells was monitored by trypan blue exclusion (Fig. 6.9 a and b). Uninfected cells and cells exposed to normal brain homogenate were included as controls and experiments were performed in duplicate. Cells exposed to scrapie brain homogenate remained viable and confluent through out the 96 hr observation period (Fig. 6.9 a and b). Uninfected cells and cells exposed to normal brain homogenate as controls also remained viable during the observation period (Fig. 6.9 a and b).

The metabolic activity of XS106 cells following exposure to the scrapie agent over a 96 hr observation period was also monitored using the Alamar blue assay which determines the chemical reduction of growth medium, as a measure of metabolic activity (Chapter 2, section 2.6.3). Uninfected XS106 cells and cells exposed to normal brain homogenate were included as a control and experiments were performed in duplicate. Experiments demonstrated that the metabolic activity of uninfected XS106 cells, cells exposed to normal brain homogenate, and cells exposed to scrapie brain homogenate remained similar and constant over the 96 hr observation period (Fig. 6.10 a and b). The metabolic activity of all cell cultures did increase at 24 hrs following addition of fresh medium at the 16 hr time point but by 48 hrs had returned to the level observed before the addition of the medium. Therefore, these data show that the addition of scrapie brain homogenate to XS106 cells does not adversely affect cell viability or cellular metabolism. Furthermore,

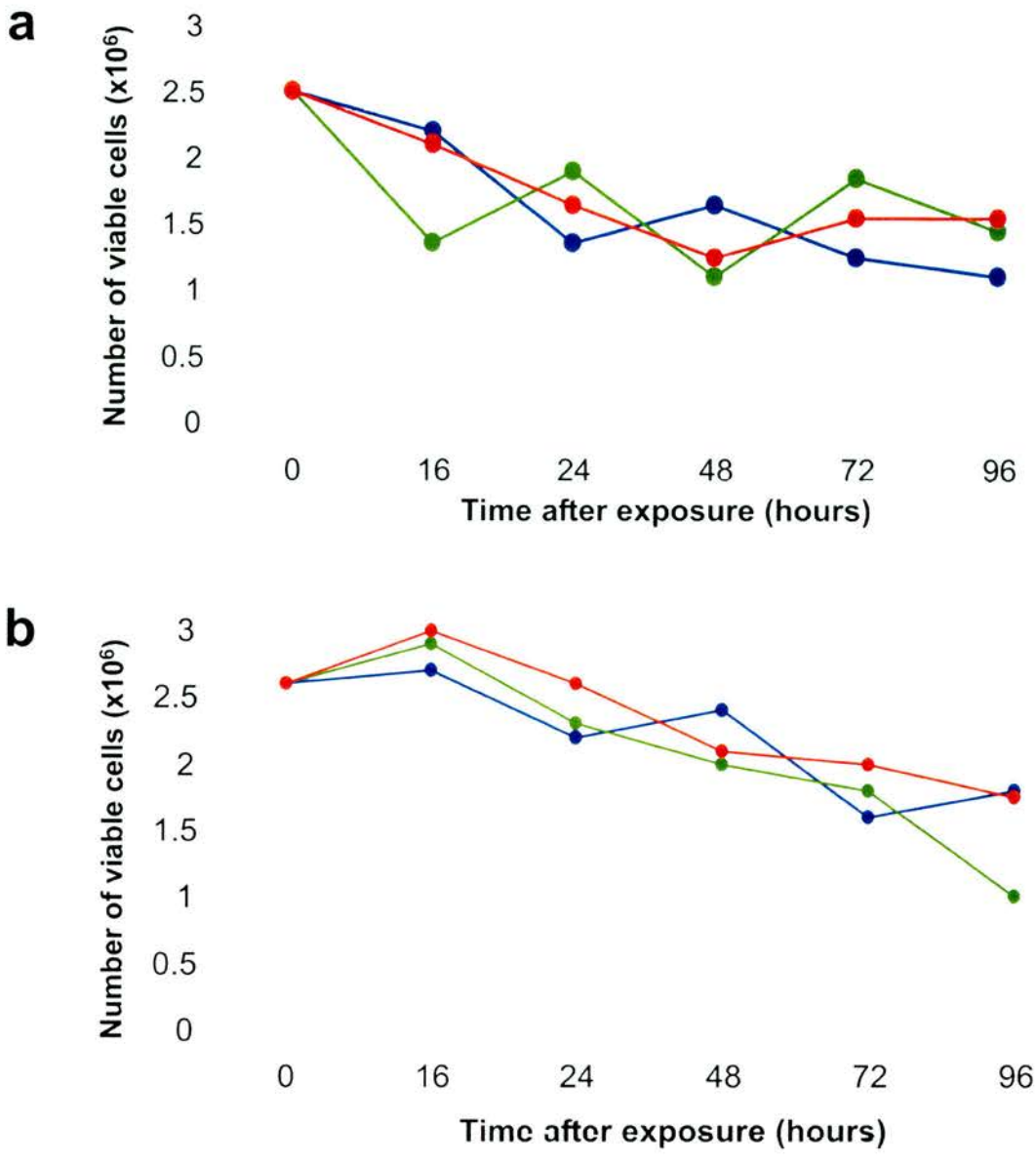


Figure 6.9- The viability of scrapie exposed XS106 cells as determined by trypan blue exclusion. All experiments were performed in duplicate (a and b). XS106 cell viability was determined following exposure to either scrapie brain homogenate (red), normal brain homogenate (green) or uninfected cells (blue). Cells were exposed to brain homogenate for 16 hrs and then maintained in culture for up to 96 hours.

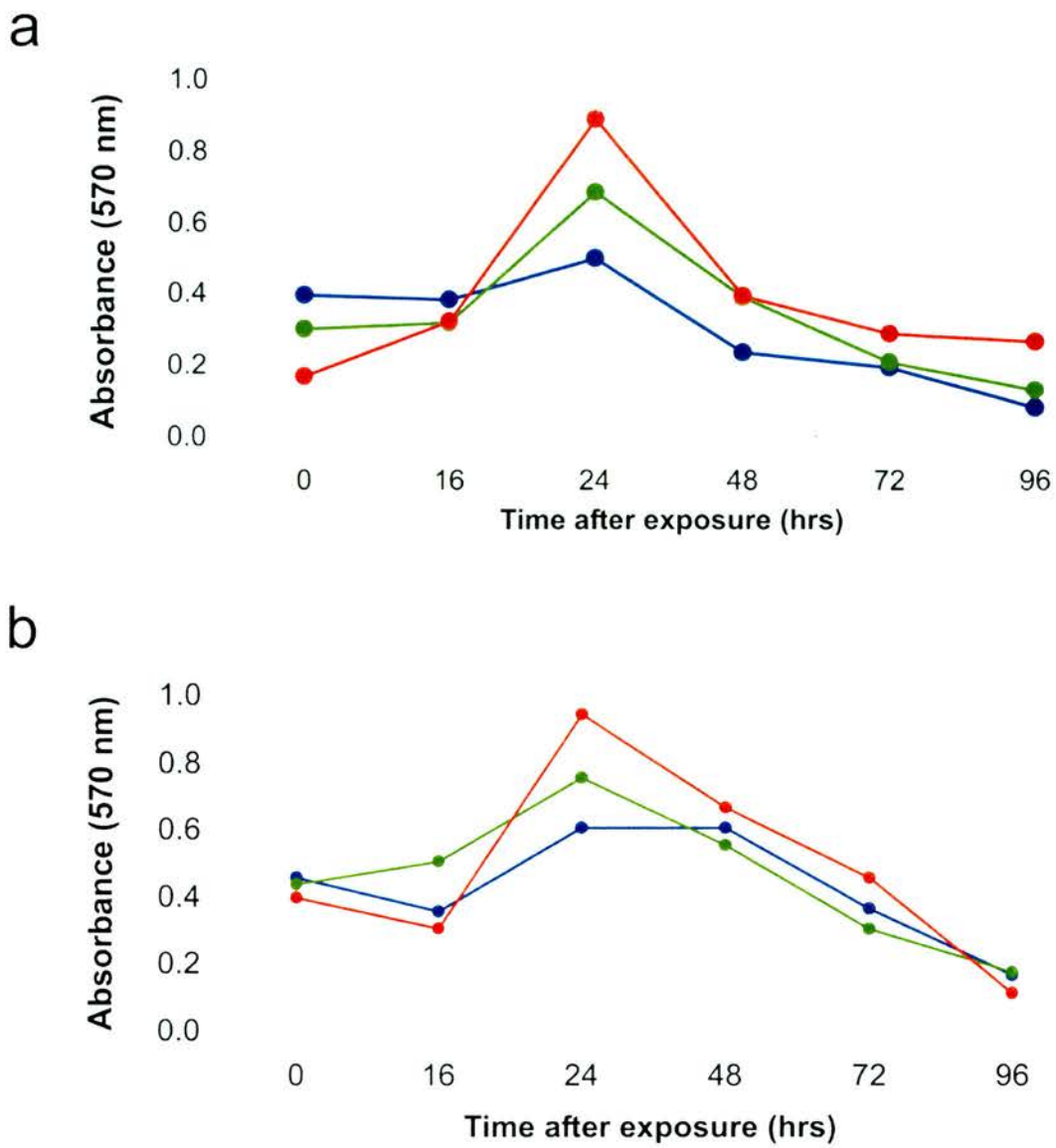


Figure 6.10- The metabolic activity of XS106 cells following exposure to scrapie as determined by the Alamar blue assay. All experiments were performed in duplicate (a and b). The metabolic activity of XS106 cells was determined following exposure to either scrapie brain homogenate (red), normal brain homogenate (green) or un-infected cells (blue). Cells were exposed to brain homogenate for 16 hrs and maintained in culture for up to 96 hours.

these data suggest that the reduction in detection of PrP^{Sc} after exposure of XS106 cells to scrapie brain homogenate was not simply due to a loss of viable cells.

6.3.8 XS106 cells reduce scrapie infectivity.

To determine whether the degradation of PrP^{Sc} by XS106 cells was concurrent to a reduction in scrapie infectivity, lysates were prepared from XS106 cells exposed to terminally scrapie-affected brain homogenate (Chapter 2; section 2.8.5) at the times indicated. XS106 cells exposed to normal brain homogenate and non-infected XS106 cells were included as negative controls. As a further control the XS52 cell line was used in parallel studies to determine whether any degradation of scrapie infectivity by XS106 cells was typical of any *in vitro* cultivated cell line exposed to the scrapie agent. After exposure to the scrapie agent at the times indicated, cell lysates were prepared and infectivity titres were determined by incubation period assay by intra-cerebral injection of 2 x 10^5 cells into each of nine C57BL/Dk indicator mice (Chapter 2; section 2.8.5). Infectivity titres are expressed as log i.c. 50% infectious dose (ID_{50})/per 1 X 10^6 cells.

To determine the starting dose of inoculum cells were initially exposed to in this chapter, an equivalent amount of scrapie-affected brain homogenate was injected i.c into a group of indicator mice. All assay mice inoculated with this homogenate alone developed clinical signs of scrapie at approximately 173 ± 4 days post-inoculation (dpi), representing an approximate scrapie infectivity titre of 6.8 log i.c. ID_{50}/dose (Table 6. 2). As expected all assay mice which were exposed to normal brain homogenate only or lysates from non-infected cells or cell lysates prepared

Table 6.2- Scrapie infectivity in cell lysates (n = 1 X 10^6) prepared from XS cell lines

Sample[a]	Time post-exposure (hrs)	Incidence[b]	Mean incubation period ± SEM (days)	titre[c]
XS106 alone	n/a	0/9	9 X > 266	n/a
XS52 alone	n/a	0/8	8 X > 266	n/a
NB alone	n/a	0/8	8 X > 266	n/a
ME7 alone	n/a	6/6	173 ± 4	6.8
XS106 + NB	16	0/9	9 X > 266	n/a
XS106 + ME7	16	8/9	190 ± 3 1 X> 266	5.9
XS106 + ME7	24	8/8	188 ± 2	6.0
XS106 + ME7	96	7/8	213 ± 7 1 X> 266	4.7
XS52 + NB	16	0/7	7 X > 266	n/a
XS52 + ME7	16	7/7	185 ± 7	6.2
XS52 + ME7	24	8/8	188 ± 1	5.3
XS52 + ME7	96	6/6	194 ± 4	5.7

[a]; Abbreviation NB = normal brain homogenate; ME7 = terminally scrapie-affected brain homogenate, XS106 = XS106 cells, XS52 = XS52 cells.

[b]; Incidence = number of animals affected/number of animals tested. The notation "N X > 266" means the mice were free of the signs of scrapie up to at least this time after inoculation.

[c]; Scrapie infectivity titres expressed as log i.c. 50% infectious dose units/1 X 10^6 cells.

from cells exposed to normal brain homogenate remained free from the signs of the disease up to 266 dpi (Table 6.2), suggesting that an infectivity titre if present would be below 2.8 log i.c. ID_{50}/1 X 10^6.

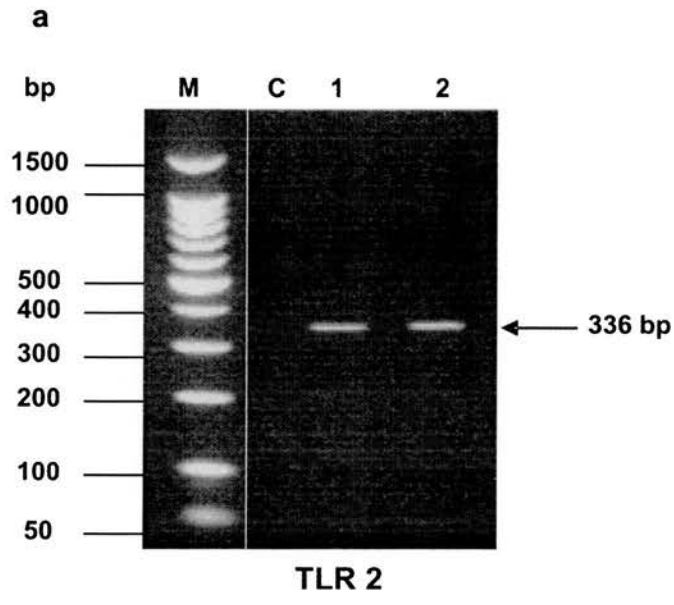
The majority of assay mice (8/9) inoculated with cell lysates prepared from XS106 cells exposed to scrapie brain homogenate collected at 16 hrs post exposure developed clinical signs of scrapie at approximately 190 ± 3 dpi, representing an approximate scrapie infectivity titre of 5.9 log i.c. ID_{50}/1 X 10^6 cells (Table 6.2). Similarly, all assay mice inoculated with XS106 cell lysates collected at 24 hrs post-exposure developed clinical signs of scrapie at approximately 188 ± 2 dpi, representing an approximate scrapie infectivity titre of 6.0 log i.c. ID_{50}/1 X 10^6 cells (Table 6.2). No significant difference between the infectivity titres at 16 hrs and 24 hrs after exposure was observed (P = 0.586) as determined by statistical analysis of disease incubation periods for each group of each indicator mice (Chapter 2; section 2.9). However, by 96 hrs after exposure the infectivity titre had declined to approximately 4.7 log i.c. ID_{50}/1 X 10^6 cells (Table 6.2) which was significantly less than the level measured at both 16 and 24 hrs post-exposure as determined by statistical analysis of disease incubation periods for each group of indicator mice (P = 0.004 and P = 0.002 respectively). In contrast the level of scrapie infectivity associated with XS52 cells at 16, 24 and 96 hrs post-exposure (range 5.7 – 6.2 log i.c. ID_{50}/1 X 10^6 cells; Table 6.2) was not significantly different at each time point (P = 0.596, 0.145 and 0.625 respectively). Furthermore, no significant difference was observed between XS52 cell lysates at 16 and 24 post-exposure when compared to XS106 cell lysates at 16 and 24 hrs post-exposure (P = 0.455 and 0.963 respectively). However, a significant difference between the level of scrapie

infectivity associated with XS52 cells at 96 hrs post-exposure and the level of infectivity associated with XS106 cells at 96 hrs post-exposure was observed (P = 0.002). Taken together these data suggest that the partial degradation of PrP^{Sc} by XS106 cells is concurrent to a partial decline in the level of scrapie infectivity associated with these cells. Furthermore, these data show that the progressive decline in scrapie infectivity observed in cultures of XS106 cells exposed to scrapie brain homogenate is not a property of all *in vitro* cell cultures.

6.3.9 LPS inhibits the degradation of PrP^{Sc} by XS106 cells

Previous research has suggested that stimulation of the innate immune system with CpG oligonucleotides shortly after peripheral exposure of mice to the scrapie agent increases survival time (Sethi et al., 2002). Therefore, experiments were performed in this chapter to investigate the effect of another stimulate of the innate immune system, LPS, on XS106 cells exposed to the scrapie agent.

LCs interacts with LPS via the toll-like receptors (TLRs) 2 and 4 (Kaisho and Akira, 2003; Takeuchi et al., 2003) in association with the surface receptor CD14 (Wright et al., 1990). To confirm that XS106 cells expressed both these TLRs their mRNA expression was determined by RT-PCR analysis. Data presented here shows that XS106 cells express both TLR-2 and TLR-4 (Fig. 6.11). The expression of CD14 was not investigated in this study however, others have shown that XS cell lines express CD14 (Yamada and Katz, 1999). Taken together these data suggest that XS106 cells would respond to LPS stimulation. Experiments demonstrated XS106



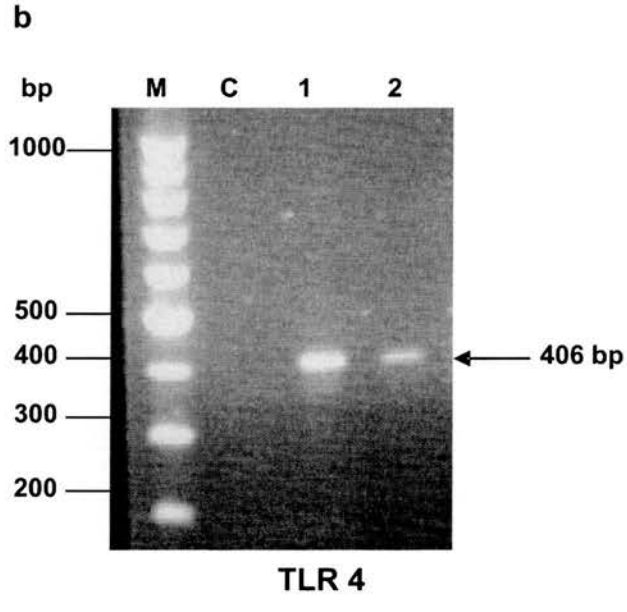


Fig 6.11- XS106 cells express mRNA for TLR 2 (a) and TLR 4 (b). Expression was determined by RT-PCR analysis. All products were resolved through gel electrophoresis containing ethidium bromide. RT-PCR analysis confirmed the presence of TLR 2 mRNA by the visualisation of a single band at 336 bp (a; lanes 1and 2). The presence of TLR 4 mRNA was confirmed by the visualisation of a single band at 406 bp (b; lanes 1 and 2). Lane M, 100 bp molecular marker. Lane C, mRNA obtained from XS106 cells as a control.

cells exposed to scrapie brain homogenate alone showed a progressive decrease in PrP^{Sc} over the 96hr observation period (Fig 6.12 c). However, cells stimulated with LPS prior to exposure to scrapie brain homogenate showed no decrease in PrP^{Sc} over the same time period (Fig. 6.12 a). In contrast, no PrP^{Sc} was detected in association with XS106 cells exposed to normal brain homogenate and LPS (Fig. 6.12 a) or cells exposed to LPS alone as controls (Fig. 6.12 e) at any of the time points analysed. Ethidium bromide staining confirmed the successful transfer of cells to the membranes in each instances (Fig. 6.12 b, d and e).

As PrP^{C} is converted to PrP^{Sc} during scrapie infection (Prusiner, 1991) FACS analysis was used to determine whether LPS stimulation altered the surface expression of PrP^{C} by XS106 cells. Experiments demonstrated that stimulation of XS106 cells with LPS did not alter their surface expression of PrP^{C} (Fig. 6.14 b). Similarly, analysis of LPS stimulated cells exposed to normal brain homogenate showed no significant difference in PrP^{C} expression in comparison to cells exposed to normal brain homogenate alone (Fig. 6.14 c). However, LPS stimulated cells exposed to scrapie brain homogenate showed a significant increase in PrP^{C} expression in comparison to cells exposed to scrapie brain homogenate only (Fig. 6.14 d; $P = 0.028$). This increase in PrP^{C} expression by LPS stimulated cells exposed to scrapie brain homogenate was not due to an increase in cell size or proliferation as no visible difference was observed between scatter plots which determine the size and complexity of cell populations (Fig 6.15 a and b).

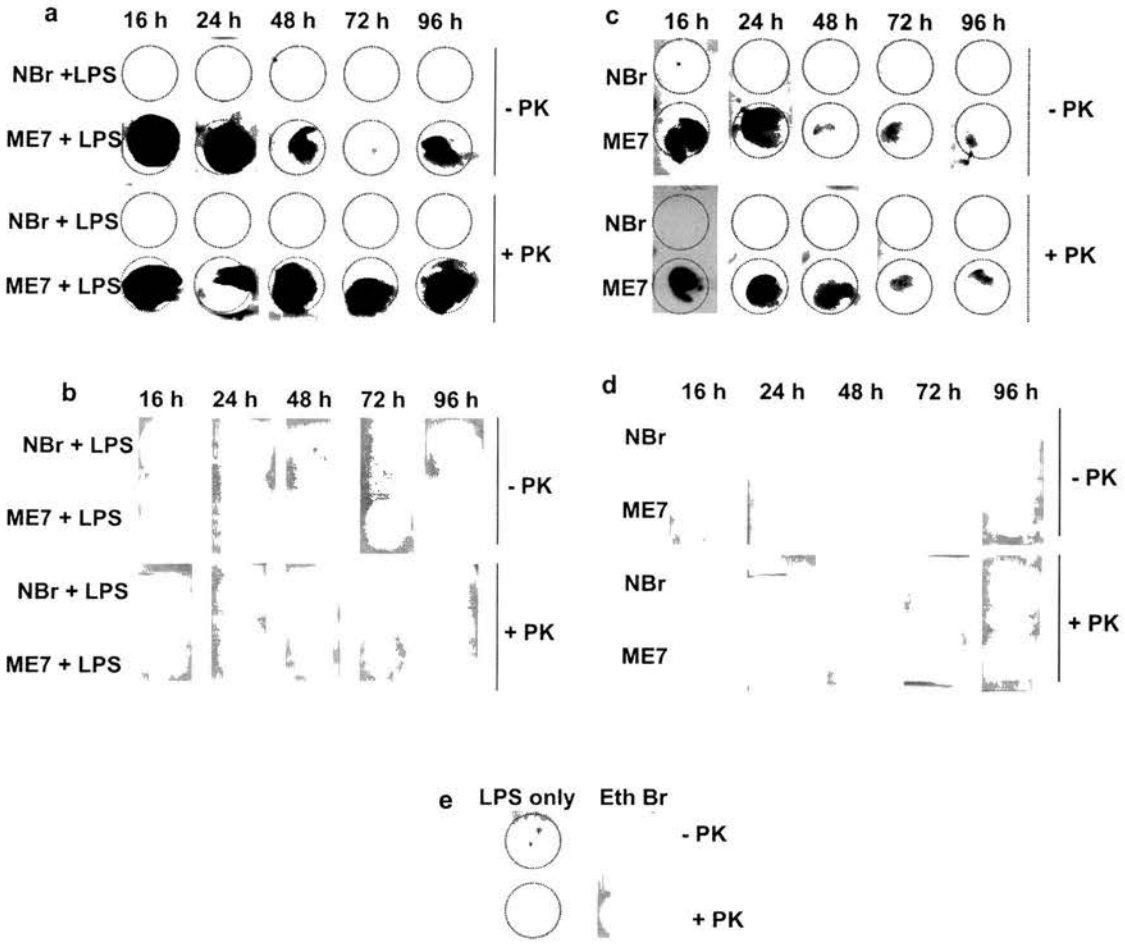


Figure 6.12- LPS stimulation blocks the degradation of PrP^{Sc} by XS106 cells. XS106 cells were stimulated with LPS one hour before exposure to scrapie brain homogenate (a). Duplicate cultures of XS106 cells were exposed to either normal brain homogenate (NBr) and LPS or scrapie brain homogenate (ME7) and LPS for 16 hrs. Cells were maintained in culture for the times indicated and then transferred to PVDF membranes and membranes treated in the absence (-) or presence (+) of proteinase K (PK). Membranes were probed with the PrP-specific monoclonal antibody 8H4. Cell blot analysis showed abundant accumulations PrP^{Sc} in association with XS106 cells over the 96 hr time period (a). In contrast XS106 cells exposed ME7 only (c) showed a progressive decrease in PrP^{Sc} over the 96 hr observation period. No PrP^{Sc} was detected in association with XS106 cells exposed to normal brain homogenate and LPS (a) or LPS only (e). Ethidium bromide (Eth Br) staining of membranes confirmed the presence of cellular DNA (b and d). This figure is representative of two separate studies.

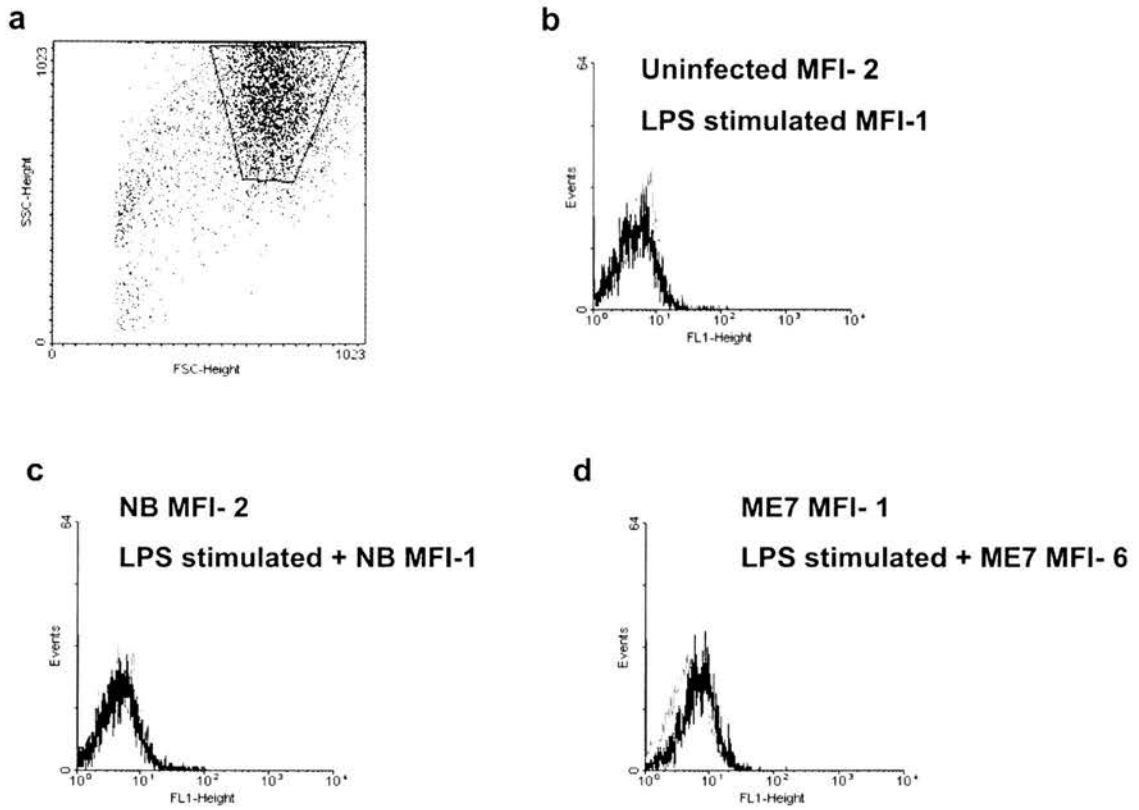


Figure 6.14- The effect of LPS stimulation on PrP^C expression by XS106 cells. XS106 cells were gated as shown (a). Uninfected vs LPS stimulated cells (b); cells exposed to normal brain homogenate (NB) vs cells stimulated with LPS and exposed to NB (c) and cells exposed to scrapie brain homogenate (ME7) vs cells stimulated with LPS and exposed to ME7 (d) were stained with the PrP-specific monoclonal antibody 8H4 and then analysised by FACS. MFI; mean fluorescent intensity of cells which stained positive for surface markers.

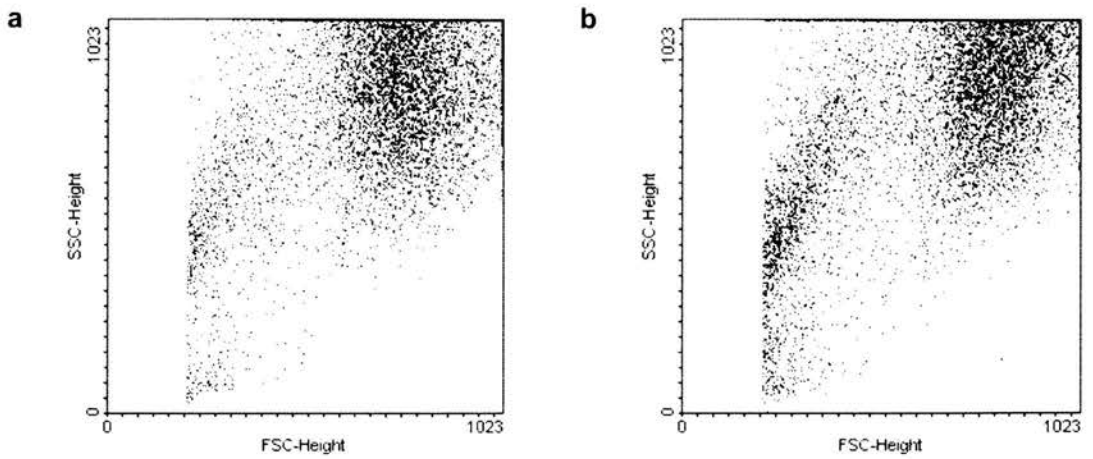


Figure 6.15- The size (FSC-height) and complexity (SSC-height) of XS106 cells was unaltered following LPS stimulation. Scatter plots of XS106 cells exposed to scrapie brain homogenate alone (a) or cells stimulated with LPS prior to scrapie exposure (b) were produced by FACS analysis.

Together these data show that LPS stimulation inhibits the degradation of PrP^{Sc} by XS106 cells exposed to the scrapie agent. The inhibited degradation of PrP^{Sc} by LPS stimulated XS106 cells might be due to the increased expression of PrP^{C} by XS106 cells following LPS stimulation.

6.3.10 The surface expression of co-stimulatory and activation markers by XS106 cells is unaltered following exposure to the scrapie agent.

XS106 cells were analysed by FACS analysis to determine whether exposure of the cells to scrapie brain homogenate altered the expression of the following surface antigens: PrP, CD40, CD80, CD86, Ia^{k}, CD205, CD54 and CD11c (Fig. 6.16 a-j). XS106 cells were exposed to scrapie brain homogenate as described in Chapter 2 (section 2.8.2) and analysed 24 hrs post-exposure. XS106 cells exposed to normal brain homogenate for the same time period were included as a reference control. All experiments were performed in quadruplicate and statistical analysis performed.

Data demonstrates that exposure to the scrapie agent did not significantly change the expression of PrP or any of the co-stimulatory or activation markers analysed when compared to cells exposed to normal brain homogenate (Fig.6.16 a-j) (Table 6.3). Therefore these data suggest that XS106 cells do not undergo maturation or activation after *in vitro* exposure to the scrapie agent.

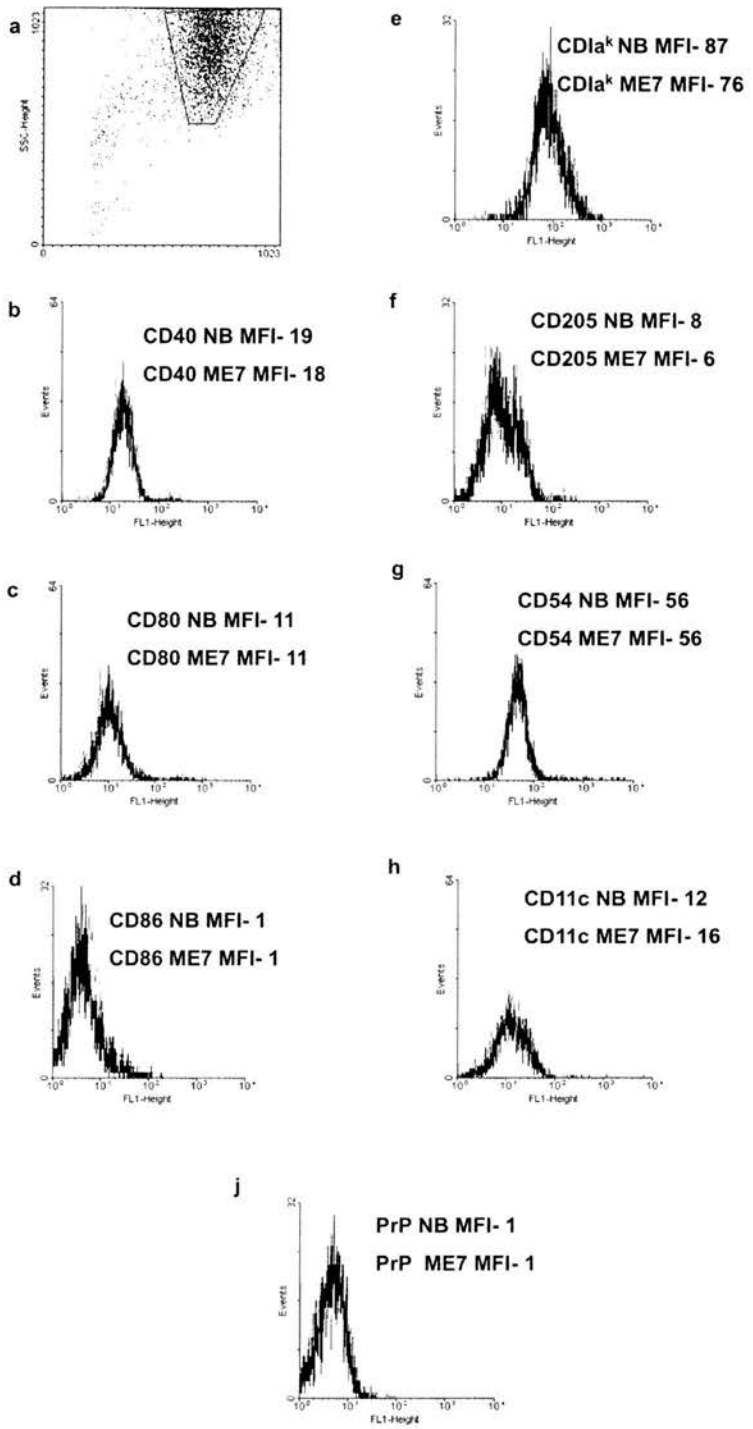


Figure 6.16- Flow cytometry (FACS) analysis of the surface expression of PrP and co-stimulatory and activation markers by XS106 cells following 16 hrs exposure to scrapie brain homogenate (ME7). XS106 cells were gated as shown (a). Cells were stained with a panel of monoclonal antibodies specific for; CD40 (b), CD80 (c), CD86 (d), Ia^k (e), CD205 (f), CD54 (g), CD11c (h) and 8H4 (PrP; i). Cells were exposed to either ME7 (black histograms) or normal brain (NB) (grey histograms). MFI; mean fluorescent intensity of cells which stained positive for surface markers.

Table 6.2- Effect of scrapie exposure on surface expression by XS106 cells. XS106 cells were exposed to either scrapie brain homogenate (ME7) or normal brain homogenate (NBr) for 16 hrs and surface antigen expression analysised at 24 hrs post-inoculation by flow cytometry. Data represents the mean fluorescent intensity (MFI) ± standard deviation (SD).

	Mean Fluorescent Intensity ± SD	
Surface Antigens	**NBr**	**ME7**
CD40	19 ± 3	18 ± 1
CD80	11 ± 1	11 ± 1
CD86	1 ± 0	1 ± 0
Ia^k	86 ± 15	75 ± 12
CD205	8 ± 2	6 ± 4
CD54	55 ± 10	56 ± 5
CD11c	11 ± 2	16 ± 2
PrP	1 ± 0	1 ± 0

6.4 Discussion

Studies by Huang *et al* demonstrated that dendritic cells were capable of acquiring and transporting PrP^{Sc} across the gut lumen (Huang et al., 2002). Furthermore the prion protein peptide $PrP_{106-126}$ is a chemoattractant (Kaneider et al., 2003) and pro-inflammatory stimulus (Bacot et al., 2003) for monocyte-derived dendritic cells. These studies suggest that LCs may also have the potential to acquire and respond to the scrapie agent following inoculation via the skin. In order to further investigate the interaction of LCs with the scrapie agent, the LC-like cell line XS106 was utilised. Data presented here shows that XS106 cells rapidly associate with scrapie brain homogenate following *in vitro* exposure. Experiments also suggest that XS106 cells partially degrade PrP^{Sc} and scrapie infectivity over a 96 hr observation period. Surprisingly, exposure to the scrapie agent did not change the surface expression of PrP or the following co-stimulatory and activation markers: CD40, CD80, CD86, Ia^k, CD205, CD54 and CD11c. Together these data suggest that LCs might partially degrade the scrapie agent within the epidermis but exposure to the agent would not induce their maturation. Interestingly LPS stimulation inhibited PrP^{Sc} degradation by XS106 cell suggesting that the ability of LCs to degrade PrP^{Sc} might be lost in the presence of other antigens within the epidermis. This loss of function might be related to the up-regulation of PrP^{C} expression on XS106 cells following LPS stimulation.

The XS106 cell line was originally established from the epidermis of newborn A/J mice (Timares et al., 1998) and has previously been characterised as having a "mature" LC phenotype due to high expression of Ia, CD80 and CD86 in comparison

to other XS cell lines using the same experimental conditions (Timares et al., 1998; Xu et al., 1995a; Xu et al., 1995b). Similarly in this study XS106 cells expressed high levels of Ia^k, CD80, CD40 and CD86 in comparison to the XS52 cells. In this study FACS analysis suggested that the expression of Ia^k, CD80 and CD86 by these cells is relatively low compared to the levels reported by others (Schuhmachers et al., 1995; Xu et al., 1995b). These differences in surface phenotype may be due to differences in the concentrations of growth factors added to the culture medium. XS106 cells are cultured in the presence of GM-CSF, which induces LC maturation and up-regulation of the expression of Ia^k, CD80 and CD86 (Romani et al., 1989; Shimada et al., 1987). These cells are also cultured in the presence of supernatant derived from the culture medium of the stromal cell line NS47 (Xu et al., 1995a). This supernatant contains colony-stimulating factor-1 which has been shown to inhibit the maturation of XS cell lines (Takashima et al., 1995). Therefore, it is possible that the relative amounts of these and other factors added to the media used in this study differed from those used in other laboratories (Timares et al., 1998). Such differences might have contributed to the differing surface phenotypes observed between studies. Furthermore, differences in the source and types of antibodies used in this study and others could contribute to the differences in phenotype observed. However, the levels of expression of the maturation markers; Ia^k, CD80, CD40 and CD86 by XS106 cells in comparison to XS52 cells used in this study suggests that XS106 cells were of a "mature" phenotype.

To maintain TSE infection, host cells must express the cellular isomer of the host prion protein, as mice deficient in PrP^C (*Prnp*$^{-/-}$ mice) do not develop disease (Bueler

et al., 1993; Manson et al., 1994a). Previous studies have suggested that LCs express PrP^C (Sugaya et al., 2002), although others have reported that the PrP^C status of DCs is variable (Luhr et al., 2002). Experiments in this chapter show that XS106 cells express both PrP^C mRNA and protein, thus suggesting that XS106 cells might be permissible to replication of the scrapie agent. However studies in this thesis demonstrate that the PrP^C status of bone-marrow derived cells, which include LCs, is not critical for scrapie pathogenesis following inoculation via the skin (Chapter 4).

XS106 cells were analysed by immunofluorescent confocal analysis to determine whether these cells were able to acquire PrP^{Sc} following *in vitro* exposure to scrapie-affected brain homogenate. Previous studies have shown that mis-folded PrP^C at the cell surface is internalised (Kiachopoulos et al., 2004) and undergoes retrograde transport to the endoplasmic reticulum were it is it is targeted for degradation (Beranger et al., 2002; Heller et al., 2003). Following exposure of XS106 cells to either scrapie brain homogenate or normal brain homogenate in this study, deposits of PrP which appeared intra-cellular could be detected within 10 mins of exposure and were maintained for the 24 hour time period studied. These PrP deposits appeared to be localised in the region of the endoplasmic reticulum and the Golgi. Recent studies utilising neuroblastoma N2a cells infected with the scrapie agent have shown that PrP^{Sc} is translocated to the nucleus (Mange et al., 2004). In this study PrP appears to accumulate in the nucleus as shown by the close association of PrP (green) with DNA (blue) (Fig. 6.4). However, it is recognised that conclusions on the apparent localisation of PrP within XS106 cells would require further confirmation as cytospin preparations as used this study can display an altered

cellular morphology. To determine the precise cellular localisation of PrP, immunofluorescent confocal analysis should be repeated on XS106 cells cultured and immunolabelled on concave coverslips in order to maintain their cellular morphology. Alternatively electron microscopy could also be used to determine the precise cellular location of PrP. Furthermore, confocal studies alone were unable to determine whether the PrP detected was PrP^{Sc} from the inoculum, or the up-regulated expression of PrP^{C} by XS106 cells, or a combination of both.

Confocal analysis of uninfected cells displayed visibly less immunostaining for PrP than that associated with cells exposed to either scrapie or normal brain homogenate. The intensity of PrP staining in uninfected cells appeared to increase slightly over the 24 hr time period studied. The function of PrP^{C} is not known, however studies have suggested that PrP^{C} may be involved in cell survival under conditions of physiological stress by functioning as an antioxidant (Martins et al., 2002). Therefore, the increased PrP staining on uninfected XS106 cells might be related to increased cellular stress due to prolonged *in vitro* culture.

Previous studies have shown that macrophages are capable of sequestering and degrading scrapie infectivity *in vitro* (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982). Studies have also shown that $CD11c^{+}$ dendritic cells *in vitro* are capable of degrading PrP^{Sc} (Luhr et al., 2002; Lupi, 2002). Thus LCs may likewise be able to acquire and degrade the scrapie agent. To determine whether the LC-like XS106 cell line was able to degrade PrP^{Sc} and scrapie infectivity cell immunoblot analysis and incubation period assays were utilised, respectively.

Experiments here demonstrate that XS106 cells were able to acquire and partially degrade PrP^{Sc} and scrapie infectivity following *in vitro* exposure. Accumulations of PrP^{Sc} and scrapie infectivity were also detected in association with the highly immature LC-like cell line, XS52. However, the levels of PrP^{Sc} and scrapie infectivity associated with this cell line remained relatively constant over the 96 hr time period studied and did not appear to be degraded. These data demonstrate that the reduced detection of PrP^{Sc} and scrapie infectivity was not simply due to the cell culture conditions. The surface phenotype of XS52 cells has been shown to be Ia^{low}, $CD86^{-}$ and E-cadherin^{+} (Xu et al., 1995a). The surface phenotype of XS52 cells has a close resemblances to freshly cultured LCs from the epidermis (Xu et al., 1995a), whereas XS106 cells have a mature surface phenotype. This suggests the ability of XS106 cells to partially degrade PrP^{Sc} and scrapie infectivity may be related to its maturation and activation status of the cells. These data demonstrate that XS106 cells are able to partially degrade the scrapie agent and suggest that LCs may be able to perform the same function *in vivo*.

The time period over which observable degradation of the scrapie agent occurred (96 hrs) suggests that scrapie infected LCs *in vivo* may undergo apoptosis in this time frame (Austyn et al., 1988). *In vivo* LCs migrate to the draining lymph node within 24 hrs of encountering antigen. Following presentation of antigen to T-lymphocytes LCs and are considered to undergo apoptosis within the draining lymph node within 3-6 days of arrival as no LCs can be detected within the efferent lymphatics (Austyn et al., 1988). Thus LCs *in vivo* might release any non-degraded residual PrP^{Sc} into

the surrounding microenvironment either in the epidermis or the draining lymph node contributing to disease pathogenesis.

The viability and cellular metabolism of the cell cultures was monitored following exposure to scrapie homogenate to determine whether the reduced levels of PrP^{Sc} detected were due to impaired cellular metabolisms or cell death. No visible difference in cell viability or metabolism was observed in scrapie exposed cells when compared to uninfected cells or cells exposed to normal brain homogenate. Similar studies have shown that scrapie infection does not affect the growth rate or cellular metabolism of N2a neuroblastoma cells when compared to uninfected controls (Bosque and Prusiner, 2000). Recent research suggests that cellular viability is only compromised in scrapie infected cells if PrP^{Sc} accumulates in the cytosol (Ma and Lindquist, 2001; Ma et al., 2002). In the studies presented here, PrP^{Sc} appeared to accumulate within the Golgi and endoplasmic reticulum of XS106 cells and not within the cytosol following exposure to scrapie brain homogenate.

LCs have been shown to become to activated and mature in response to different exogenous stimuli including, inflammatory cytokines (Cumberbatch et al., 2001; Cumberbatch et al., 1997b), CpG oligonucleotides (Ban et al., 2000) and LPS (De Smedt et al., 1996). Studies by Sethi *et al* (Sethi et al., 2002) have shown that treatment of scrapie infected mice with CpG oligonucleotides extends the disease incubation period. The effect of CpG treatment on scrapie disease pathogenesis was suggested to be due to the stimulation of the innate immune system, possibly via TLRs (Sethi et al., 2002). Studies have demonstrated that LCs express TLR-2 and

TLR-4, which both bind LPS (Kaisho and Akira, 2003) and data presented in this chapter shows that XS106 cells also express both TLR-2 and TLR-4. To investigate whether LPS-stimulation would increase the ability of XS106 cells to degrade PrP^{Sc}, cells were stimulated with LPS prior to exposure to scrapie-affected brain homogenate. Experiments demonstrated that LPS-stimulation blocked the degradation of PrP^{Sc} by XS106 cells. These data appear to be in opposition to the hypothesis that stimulation of the innate immune system via TLRs would increase PrP^{Sc} degradation (Sethi et al., 2002). However, recent data shows that the CpG nucleotide treatment regime carried out by Sethi *et al* (Sethi et al., 2002) destroys lymphoid follicles (Heikenwalder et al., 2004), the main peripheral site of replication for the scrapie agent (Mabbott et al., 1998). Furthermore, although exposure of dendritic cells to LPS can induces the up-regulation of TLRs 2, 4 and 9 (An et al., 2002), it is unlikely that TLRs are involved in degradation of the scrapie agent as previous studies by Prinz *et al* have shown that scrapie pathogenesis is unaffected in *Myd88*$^{-/-}$ mice (Adachi, 1998) which are defective in TLR signalling (Prinz et al., 2003a). In addition, although XS106 cells express mRNA for TLR-2 and TLR-4 recent data suggests that TLR-2 is only weakly expressed by LCs (Takeuchi et al., 2003). Therefore, it is conceivable that XS106 cells might not become fully activated following LPS stimulation.

Data presented in this chapter has shown that exposure of XS106 cells to either scrapie brain homogenate alone or LPS alone has no effect on the expression of cellular PrP^{C}. However, exposure to scrapie brain homogenate and LPS results in a significant up-regulation of PrP^{C} by these cells. If PrP^{C} is involved in cell survival

during physiological stress (Brown et al., 1997a; Brown et al., 1997b; Milhavet et al., 2000; Wong et al., 2001) it is conceivable that exposure to one of these stimuli alone would have no effect on PrP^C expression, but both stimuli together might induce physiological stress, resulting in an up-regulation of PrP^C. As PrP^C is converted to PrP^{Sc} during TSE disease (Prusiner, 1982) the up-regulation of PrP^C by host cells might enhance scrapie pathogenesis. Thus the up-regulation of PrP^C by XS106 cells following LPS stimulation and exposure to the scrapie agent might mediate the replication of PrP^{Sc}, blocking PrP^{Sc} degradation by these cells. Previous studies in mice have demonstrated that treatment with LPS prior to inoculation with the scrapie agent increase the efficiency of scrapie infection (Kimberlin and Walker, 1990). Taken together these data suggest that LPS-stimulated LCs *in vivo* might enhance scrapie pathogenesis following transmission via the skin.

Macrophages have been previously suggested to play a dual role in scrapie pathogenesis. They are considered to play a role in the degradation of scrapie infectivity (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982) but in some circumstances may also accumulate scrapie infectivity, especially following exposure to high doses of inoculum (Prinz et al., 2002). Data presented in this chapter suggest that LCs might have a similar role in TSE pathogenesis. Experiments here demonstrated that that LCs can degrade PrP^{Sc} however data also shows that these cells can retain PrP^{Sc} for example following LPS-stimulations.

The surface expression of Ia^k, CD40, CD80, CD86 and CD54 (ICAM-1) is up-regulated on LCs following antigen encounter (Cumberbatch et al., 1992; Inaba et

al., 1994; Schuler and Steinman, 1985). Studies by Bacot *et al* have shown that the prion protein peptide $PrP_{106-126}$ also induces up-regulation of the surface antigens, HLA-DR, CD40, CD80 and CD83 by human monocyte-derived DC (Bacot et al., 2003). The third question posed in this chapter was whether exposure of XS106 cells to the scrapie agent would induce a change in the surface expression of LC co-stimulatory and activation markers when compared to cells exposed to normal brain homogenate. However, the experiments presented here demonstrate that exposure to the scrapie agent did not significantly change the expression of the surface markers; CD40, CD80, CD86, Ia^k, and CD54.

The surface expression of the C-type lectin CD205 and the αX intergrin CD11c was also analysed following exposure of XS106 cells to the scrapie agent. It was hypothesised that CD205 may be involved in the internalisation of the scrapie agent, as this lectin binds carbohydrate residues and delivers them to the lysosome or late endosome where they are degraded (Figdor et al., 2002; Jiang, 1995), the same cellular compartment proposed for the degradation of PrP^{Sc} (Beranger et al., 2002; Caughey et al., 1991; Heller et al., 2003). Similarly CD11c binds complement component iC3b (Bilsland et al., 1994). Complement component C3 has been previously shown to play a role in localising the scrapie agent to lymphoid follicles (Klein et al., 2001; Mabbott et al., 2001). However, the surface expression of CD205 or CD11c did not significantly change following exposure to the scrapie agent. Expression of PrP^C by XS106 cells was also unaltered following exposure to the scrapie agent.

Scrapie infection does not illicit a humoral or cell-mediated immune response *in vivo* (Fraser and Dickinson, 1978; Fraser and Farquhar, 1987; Porter et al., 1973). In this study, the absence of changes in surface expression of co-stimulatory and activation markers on XS106 cells suggest that the scrapie agent would be unlikely to activate or induce the maturation of LCs *in vivo*. Other studies have demonstrated changes in surface expression of some of the above markers following exposure of monocyte-derived DCs to the prion protein fragment $PrP_{106-126}$ (Bacot et al., 2003). However, it is possible that the absence of change in surface expression of the above markers in this study might be due to differences in the source of the cells or the inoculum. Furthermore, the prion protein fragment used in the Bacot study (Bacot et al., 2003) is likely to lack important additional constituents of PrP^{Sc} or the TSE agent which may help to aid immune evasion.

In conclusion data presented in this chapter demonstrate that the LC-like cell line, XS106 is capable of acquiring and partially degrading PrP^{Sc} and scrapie infectivity. These data suggest that LCs *in vivo* may also be involved in the degradation of the scrapie agent after inoculation via the skin. The absence of changes in surface expression of co-stimulatory and activation markers suggest that LCs would not become activated or undergo maturation after exposure to scrapie agent. Furthermore, as the degradation of PrP^{Sc} by XS106 cells is blocked after LPS stimulation this suggests the activation of LCs by other antigens or stimuli may likewise block the ability of these cells to degrade the scrapie agent.

7

Scrapie neuroinvasion after inoculation via the skin is independent of migratory Langerhans cells

7.1 Abstract

Following peripheral exposure, TSE agents usually accumulate in lymphoid tissues before spreading to the brain. The mechanisms by which the scrapie agent is transported to lymphoid tissues after skin scarification are not known. Langerhans cells (LCs) reside in the epidermis and migrate to the draining lymph node (DLN) after encountering antigen. To investigate the potential role of LCs in the active transportation of the scrapie agent from the skin, mouse models were utilised in which their migration was inhibited either due to CD40 ligand-deficiency ($CD40L^{-/-}$ mice), or following caspase-1 inhibition. Data shows that the early accumulation of scrapie infectivity in the DLN and subsequent neuroinvasion was not impaired in mice with impaired active LC migration. Thus, active LC migration is not involved in the transport of the scrapie agent from the skin however these studies did not address LC migration in the steady-state and thus this method of transportation can not be excluded. Following intra-cerebral inoculation with the scrapie agent, wild-type mice and $CD40L^{-/-}$ mice develop clinical disease with similar incubation periods. However, after inoculation via skin scarification $CD40L^{-/-}$ mice develop disease significantly earlier than wild-type mice. The shorter incubation period in $CD40L^{-/-}$ mice was unexpected and suggests that a CD40L-dependent mechanism is involved in impeding scrapie pathogenesis. These data suggest that LCs are not involved in the active transportation of the scrapie agent from the skin to the draining lymphoid tissues, however a CD40L-dependent mechanism may be involved impeding scrapie pathogenesis in the periphery.

7.2 Introduction

Previous data in this thesis (Chapters 4 and 5) has shown that mature PrP^{C}-expressing follicular dendritic cells (FDCs) are critical for the propagation of scrapie infectivity from the periphery to the CNS after skin scarification. However, it is not known how scrapie infectivity is initially transported from the skin to FDCs within germinal centres in which it replicates. FDCs could directly trap cell-free PrP^{Sc}, or other agent associated molecules, in a complement-bound complex (Klein et al., 2001; Mabbott et al., 2001), but it is also possible that migratory cells transport the agent to lymphoid follicles.

Several cells have the potential to transport TSE agents including macrophages and migratory bone-marrow derived dendritic cells (DCs). The evidence that macrophages destroy infectivity and degrade PrP^{Sc} makes them an unlikely and inefficient transport candidate (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982). Migratory bone-marrow derived DCs are a distinct lineage from tissue-fixed, stromal derived, FDCs that are not considered to be of haemopoietic origin (Endres et al., 1999; Kapasi et al., 1993; Kaspasi et al., 1998). These DCs continually circulate throughout the host's tissues and tissue fluids where they sample antigens and transport them to lymphoid tissues (Banchereau et al., 2000). Unlike macrophages, DCs can retain some protein antigens in native, non-degraded form (Wykes et al., 1998). Furthermore, a sub-population of these migratory DCs has been shown to transport intestinally injected PrP^{Sc} to mesenteric lymph nodes via the lymph (Huang et al., 2000) but direct demonstration of their involvement in TSE pathogenesis is lacking.

Langerhans cells (LCs) are a subset of DCs that reside in the epidermis and migrate to the draining lymph node (DLN) following antigen encounter (Banchereau et al., 2000). LCs are considered to provide a potential mechanism for the transmission of some pathogens from the skin, such as dengue virus (Wu et al., 2000), HIV (Reece et al., 1998), maedi-visna lentivirus (Ryan et al., 2000) and venezuelan equine encephalitis virus (MacDonald and Johnston, 2000). These characteristics suggest that LCs are plausible candidates for the transportation of the scrapie agent from the skin to the DLNs. Furthermore, studies in this thesis have shown that LC are capable of sequestering PrP^{Sc} and scrapie infectivity (Chapter 6), further suggesting that LC might be involved in transportation of the scrapie agent to the DLNs.

LCs require a number of stimuli to induce their mobilization out of the skin (Banchereau et al., 2000). CD40 ligand (*Tnfsf5*) and its receptor CD40 (*Tnfrsf*) are members of the tumour necrosis superfamily (Banchereau et al., 1994). The CD40-CD40L signalling pathway is just one component in a complex network of stimuli that regulate the migration of antigen-bearing LCs out of the epidermis to the DLNs (Moodycliffe et al., 2000). In mice deficient in CD40 ligand ($CD40L^{-/-}$ mice), the number and morphology of LCs in the epidermis is the same as wild-type mice but they fail to migrate from the skin and substantially fewer accumulate in the DLN (Moodycliffe et al., 2000). Likewise, caspase-1 plays an important role in the regulation of LC migration. Caspase-1 is a cysteine protease which specifically cleaves the IL-1β precursor to release the biologically active cytokine (Garcia Calvo et al., 1998; Thornberry, 1991; Thornberry et al., 1992; Thornberry et al., 1994). Specific inhibition of the cytokine IL-1β by a caspase-1 inhibitor impairs LC

migration from the epidermis (Antonopoulos et al., 2001). If LCs are involved in the transportation of the scrapie agent from the skin then delaying or preventing the migration of LCs to the DLNs may result in a prolonged disease incubation period or reduced disease susceptibility.

In order to investigate the potential role of LCs in the active transportation of the scrapie agent from skin to draining lymphoid tissues, scrapie pathogenesis was studied in models where the active migration of these cells from the epidermis was impaired, either due to CD40L deficiency (Moodycliffe et al., 2000), or caspase-1 inhibition (Antonopoulos et al., 2001).

7.3 Results

7.3.1 Effect of caspase-1 inhibition on the accumulation of the scrapie agent in lymphoid tissues.

Previous studies have shown that topical treatment with a pharmacological inhibitor caspase-1 potently inhibits LC migration from the skin (Antonopoulos et al., 2001). Therefore experiments were conducted to investigate whether the early accumulation of the scrapie agent in DLNs was affected after topical treatment with a caspase-1 inhibitior. Prior to inoculation with the scrapie agent by skin scarification (1.0% scrapie brain homogenate), C57BL/Dk mice were treated at the site of inoculation with Ac-YVAD-cmk, a cell-permeable, irreversible, non-competitive caspase-1 inhibitor (Garcia Calvo et al., 1998; Thornberry, 1991; Thornberry et al., 1994), according to a previously established protocol (Antonopoulos et al., 2001) . This treatment effectively blocks both spontaneous and induced LC migration from the epidermis (Antonopoulos et al., 2001) (Fig. 7.1). As a control, application of Ac-DEVD-cmk, a specific inhibitor of caspase-3 which has little effect on caspase-1 (Garcia Calvo et al., 1998) was included. Caspase inhibitors were dissolved in dimethyl sulfoxide (DMSO) to allow absorption through the skin. Other control treatments included DMSO only and no pre-treatment prior to skin scarification.

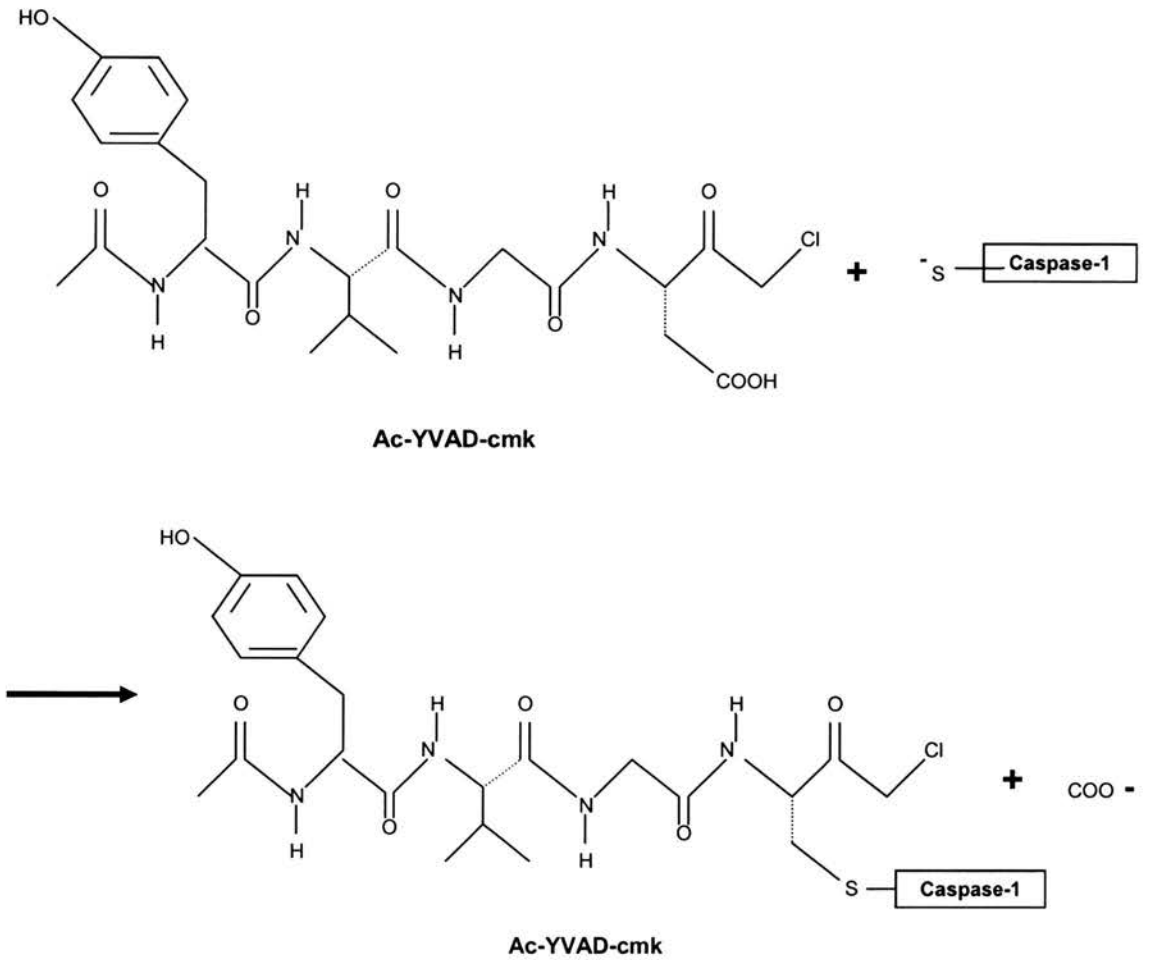


Figure 7.1- Casapse-1 inhibitor II (Ac-YVAD-cmk) specifically and irreversibly inhibits capase-1 activity by the expulsion of the carboxylate leaving group (COO^-) to form a thiomethyl ketone with the active site of the enzyme, cysteine^{285} (-S).

After peripheral inoculation of immunocompetent mice with the ME7 scrapie strain, high levels of infectivity and PrP^{Sc} accumulate in lymphoid tissues within the first few weeks post-inoculation and are maintained throughout the course of infection (Brown et al., 1999; Mabbott et al., 2000b)(Chapter 3; Figure 3.1). In the present study inguinal lymph nodes (ILNs) draining the site of inoculation were taken from mice from each treatment group 49 days after inoculation with the scrapie agent.

The scrapie infectivity titres in pooled (n = 2) tissue homogenates were estimated by bioassay in groups of up to 12 indicator mice. As expected, ILNs from untreated mice contained high levels of scrapie infectivity (approximately 6.7 log i.c. 50% infectious dose (ID_{50})/g). However, this study demonstrates that infectivity titres in ILNs from Ac-YVAD-cmk pre-treated mice were similar to those from untreated mice (approximately 6.4 log i.c. ID_{50}/g) indicating that pharmacological blockade of caspase-1 dependent LC migration from the skin did not affect the early accumulation of scrapie infectivity in ILNs. No significant effect on the early accumulation of scrapie infectivity in ILNs was observed in mice pre-treated with Ac-DEVD-cmk or DMSO alone as controls, when compared to untreated control mice (approximately 6.0 and 5.9 log i.c.ID_{50}/g, respectively).

Similarly, immunoblot analysis of pooled ILNs (n = 2) taken from terminally scrapie- affected untreated control C57BL/Dk mice detected abundant accumulations of detergent insoluble proteinase K (PK) resistant PrP^{Sc} (Fig. 7.2 a lane 2). A typical three-banded pattern was observed between the molecular mass values of 20-30 kDa, representing the unglycosylated, monoglycosylated and diglycosylated isomers of PrP (in order of increasing molecular mass). Abundant levels of PrP^{Sc} accumulation were also observed in pooled ILNs samples from mice treated with Ac-YVAD-cmk, Ac-DEVD-cmk or DMSO alone (Fig. 7.2 a lanes 4, 6 and 8). Likewise, immunoblot analysis of spleen tissue taken from terminally scrapie-affected mice showed abundant accumulation of PrP^{Sc} in tissues taken from each treatment and control groups (Fig. 7.2 b lanes 2, 4, 6, and 8). These data demonstrate that pharmacological

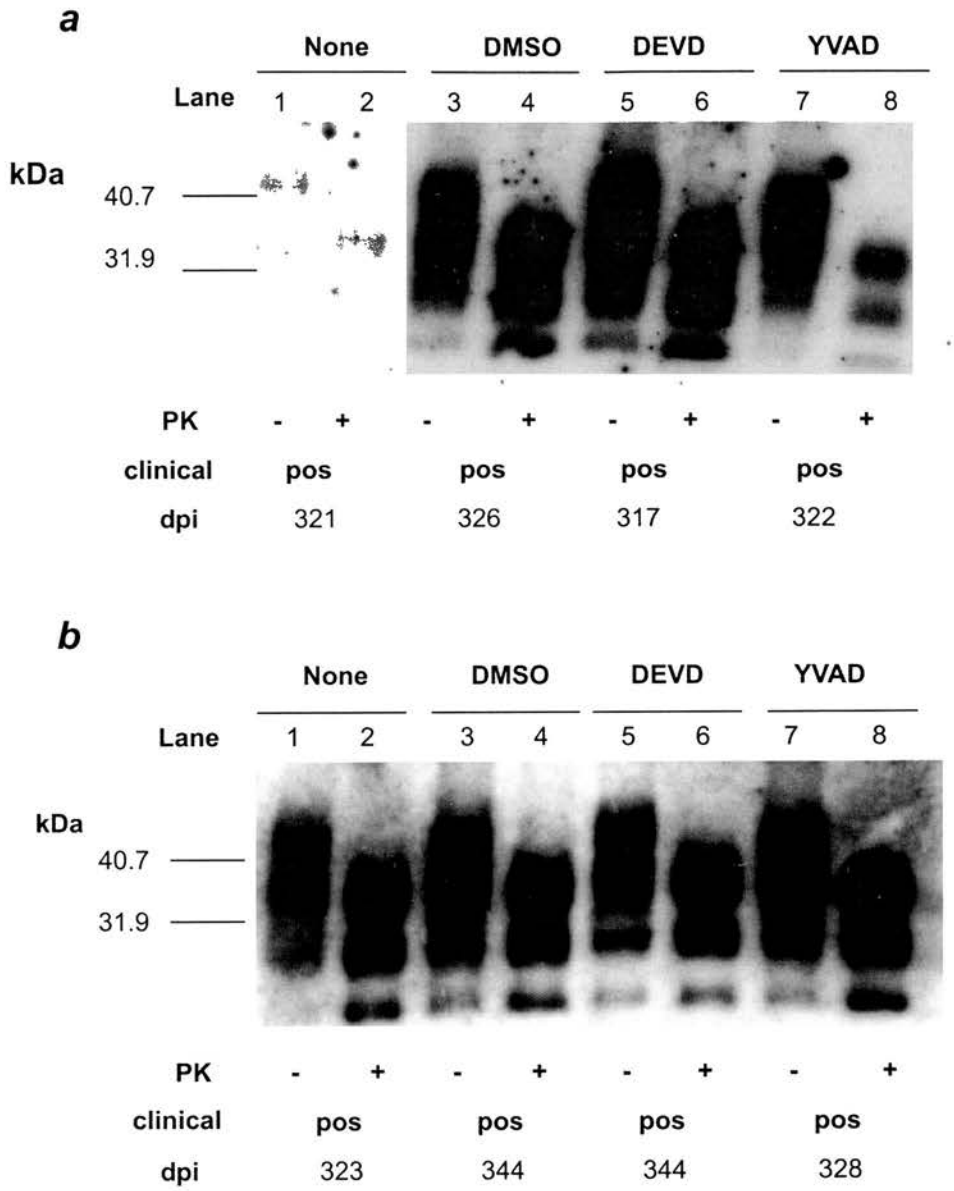


Figure 7.2– PrP^{Sc} accumulation in lymph nodes (a) and spleen tissue (b) from terminally scrapie-affected C57BL/Dk mice treated with either Caspase-1 inhibitor II (Ac-YVAD-cmk), Caspase-3 inhibitor III (Ac-DEVD-cmk) or Dimethyl sulfoxide (DMSO) prior to inoculation by skin scarification. Age-matched controls which received no treatment prior to inoculation by skin scarification were also included (None). Samples were treated in the absence (-) or presence (+) of proteinase K (PK) prior to electrophoresis. Abundant levels of PrP^{Sc} accumulation were detected in the lymph nodes (a; $n = 2$) and spleens (b) of all terminally scrapie affected mice from each treatment group. pos., mice that developed clinical signs of scrapie; dpi, days post-inoculation at which tissues were collected for analysis.

blockade of caspase-1 dependent LC migration does not effect the accumulation of scrapie infectivity or PrP^{Sc} within lymphoid tissues.

7.3.2 Effect of caspase 1 inhibition on susceptibility to scrapie infection

After inoculation by skin scarification all untreated C57BL/Dk mice in this study developed clinical scrapie with a mean disease incubation period of approximately 316 ± 2 days post-inoculation (Table 7.1). Pre-treatment with Ac-YVAD-cmk prior to inoculation with the scrapie agent had no significant effect on the onset of clinical symptoms or the disease incubation period (Table 7.1) when compared to control mice, as all mice succumbed to scrapie approximately 320 days post-inoculation (P = 0.192) (Table 7.1). No significant effect on the disease incubation period was also observed in mice pre-treated with Ac-DEVD-cmk or DMSO alone as a control when compared to untreated control mice (P = 0.774 and P = 0.148, respectively) (Table 7.1). Thus, these data show that pharmacological blockade of caspase-1 dependent LC migration does not affect susceptibility to scrapie infection when inoculated via the skin. Histopathological analysis of brain tissue from each treatment or control group displayed the characteristic spongiform pathology, gliosis and disease-specific PrP accumulation typical of peripheral inoculation with the ME7 scrapie strain (Fig. 7.3). No significant difference in the pathological targeting of vacuolation in the brain was observed between treatment and control groups (Fig. 7.4 a-d). Together these data demonstrate that pharmacological blockade of caspase-1 dependent LC migration does not affect neuroinvasion or the pathological targeting of vacuolation in the brain. These data are consistent with the finding that impaired LC migration

TABLE 7.1- Treatment of skin with the caspase-1 inhibitor II Ac-YVAD-cmk prior to scapie inoculation by skin scarification does not affect disease susceptibility.

Pre-treatment[a]	**Incidence[b]**	**Mean incubation period (days) ± SEM**
None	9/9	316 ± 2
DMSO	10/10	323 ± 4
Ac-YVAD-cmk	14/14	323 ± 4
Ac-DEVD-cmk	10/10	316 ± 3

[a], Prior to scrapie inoculation mice were pre-treated at the shaved inoculation site with Ac-YVAD-cmk (a caspase-1 inhibitor), or Ac-DEVD-cmk (a caspase-3 inhibitor) or vehicle alone (DMSO) as controls. Age-matched controls which received no treatment were also included (None).

[b], Incidence – number of animals affected/ number of animals inoculated.

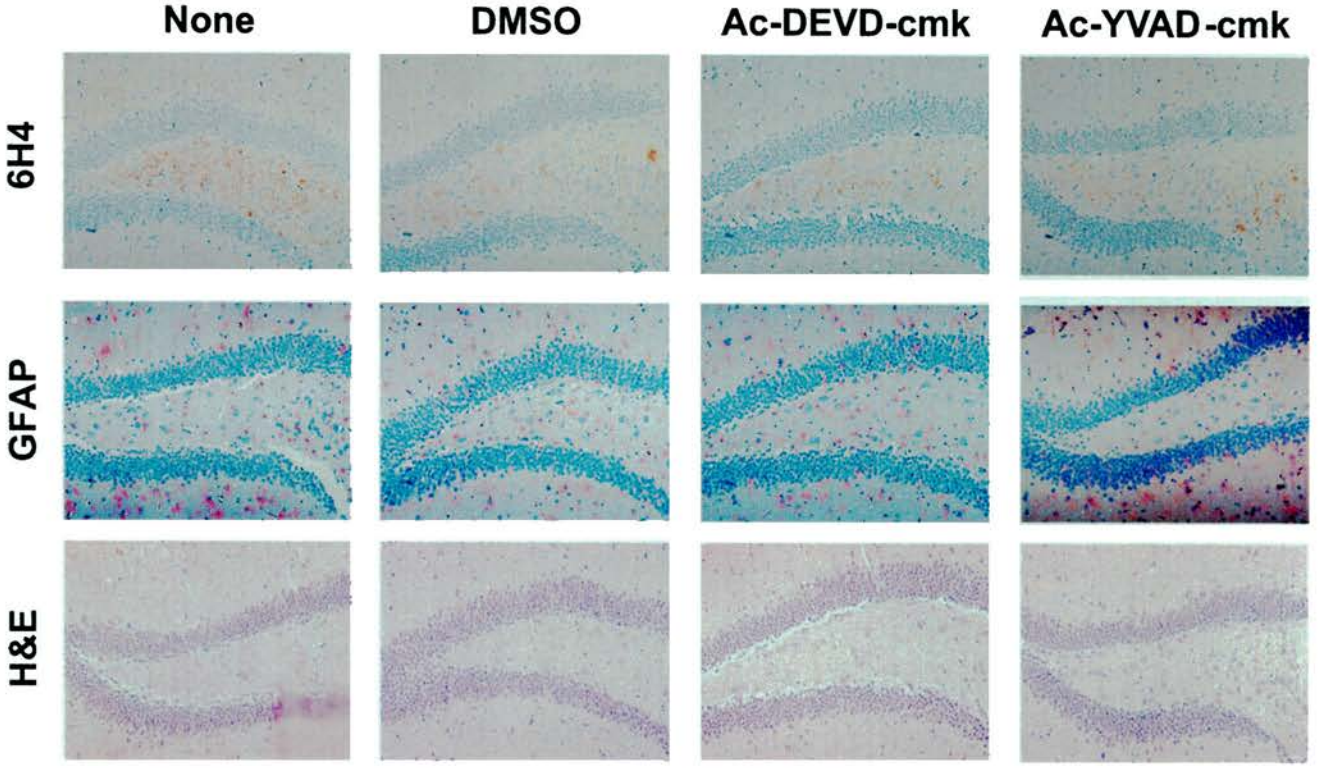


Figure 7.3- Histological analysis of brain tissue from terminally scrapie affected C57BL/Dk mice treated with Caspase-1 inhibitor II (Ac-YVAD-cmk; fourth column), Caspase-3 inhibitor III (Ac-DEVD-cmk; third column) or dimethyl sulfoxide (DMSO; second column) prior to inoculation by skin scarification. Age matched control mice which received no treatment prior to inoculation by skin scarification were also included (None; first column). Large PrP accumulations (brown) were detected in the hippocampi of all mice which developed clinical signs of scrapie (first row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP; red) shows diffuse gliosis in the hippocampi of all terminally scrapie affected mice (second row). Adjacent sections were also stained with hematoxylin and eosin and all displayed extensive vacuolation (third row). All sections were counterstained with hematoxylin (blue). Original magnification X200.

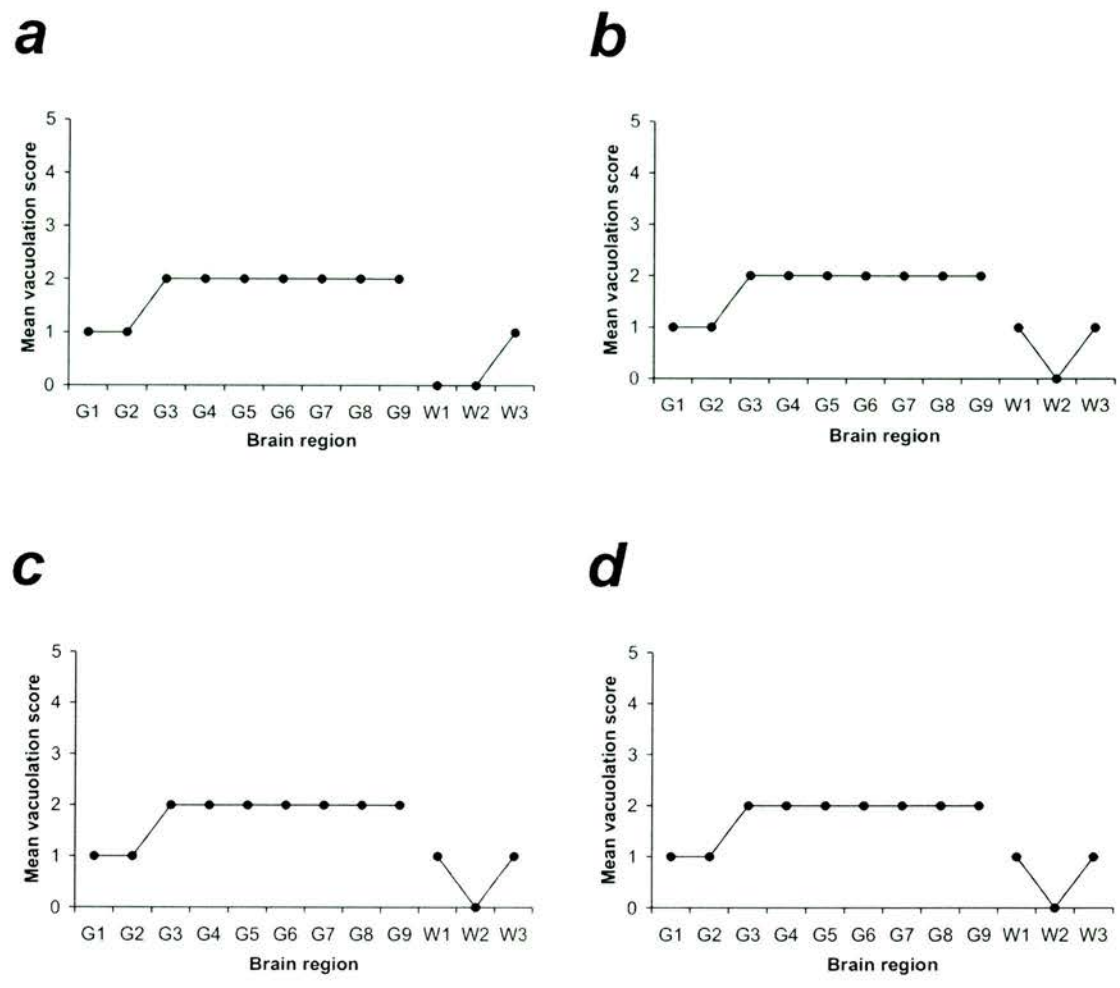


Figure 7.4- Treatment with the caspase-1 inhibitor (Ac-YVAD-cmk) prior to inoculation with scrapie strain ME7 by skin scarification does not effect pathological targeting of vacuolation. Prior to inoculation with scrapie via skin scarification mice were treated at the site of inoculation with either the caspase-1 inhibitor II, Ac-YVAD-cmk (a), Dimethly sulfoxide (DMSO) (b), or the caspase-3 inhibitor III Ac-DEVD-cmk (c). Age-matched controls which received no treatment prior to skin scarification were also included (None) (d). Vacuolation in the brain was scored on a scale of 0-5 in the following grey-matter (G1-G9) and white-matter (W1-W3) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, Decoction of superior cerebellar peduncles; W3, Cerebral peduncles. Each point represents mean vacuolation score ± S.E.M for groups of 5-9 mice.

due to caspase-1 inhibition did not affect the early accumulation of the scrapie agent within lymphoid tissues (section 7.3.1).

7.3.3 Lymphoid tissues of CD40L$^{-/-}$ mice contain FDCs but lack germinal centres.

Next scrapie pathogenesis in mice with a permanent block in LC migration out of the skin due to a targeted deletion of the *Tnfsf5* gene (CD40L$^{-/-}$ mice) was investigated (Moodycliffe et al., 2000).

Tail snips were taken from all mice used in the CD40L$^{-/-}$ study and the genotype of each mouse strain was determined by PCR analysis of total DNA (Fig. 7.5). Analysis of total DNA from wild-type mice confirmed the presence of the *Tnfsf5* gene (CD40L) by the visualization of a single band at 250 bp (Fig.7.5 lanes 1-4). In contrast analysis of DNA from CD40L$^{-/-}$ mice confirmed the absence of the wild-type allele and only the presence of a band specific for the neomycin resistance gene at 500 bp (Fig. 7.5 lanes 5-8). These data confirmed the genotypes of all mice used within this study were correct.

CD40L$^{-/-}$ mice are incapable of mounting T-cell dependent humoral immune responses and as a consequence germinal centre development is impaired (Xu et al., 1994). Therefore, FDC status was assessed in spleens from CD40L$^{-/-}$ mice and C57BL/6 wild-type mice. Germinal centres containing PNA-binding B lymphocytes were detected in the spleens of all C57BL/6 wild-type mice, but not in spleens of CD40L$^{-/-}$ mice (Fig. 7.6 a). Despite the impaired germinal centre formation in

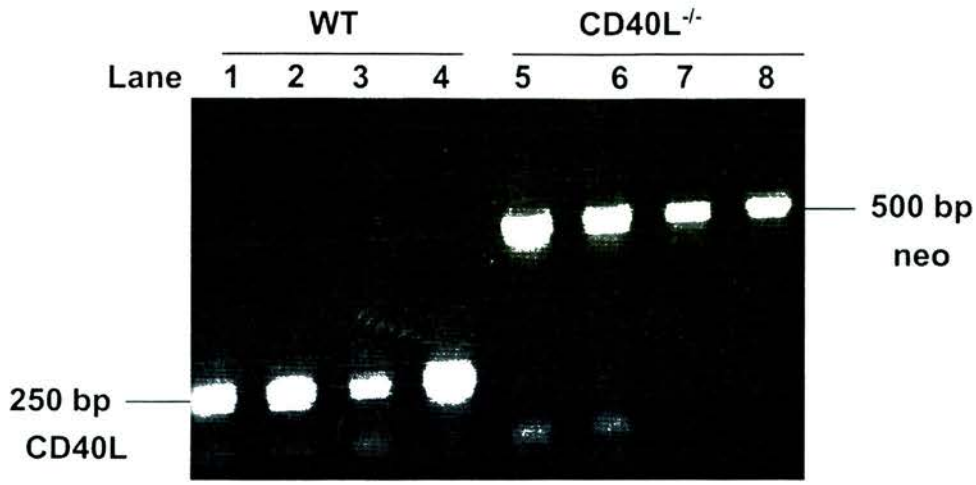


Figure 7.5- Confirmation of the presence or absence of the CD40L gene in wild-type C57BL/6 (WT) and CD40L$^{-/-}$ mice by PCR analysis of total DNA from tail biopsies. Analysis of total DNA from CD40L$^{-/-}$ mice (lanes 5-8) confirmed the presence of a portion of the neomycin resistance gene (neo) by the visualisation of only a single band at 500 bp. The presence of the CD40L allele in the WT mice (lanes 1-4) was confirmed by the visualisation of only a single band at 250 bp. Results of four mice from each genotype are shown which are representative of results from all mice used in this study.

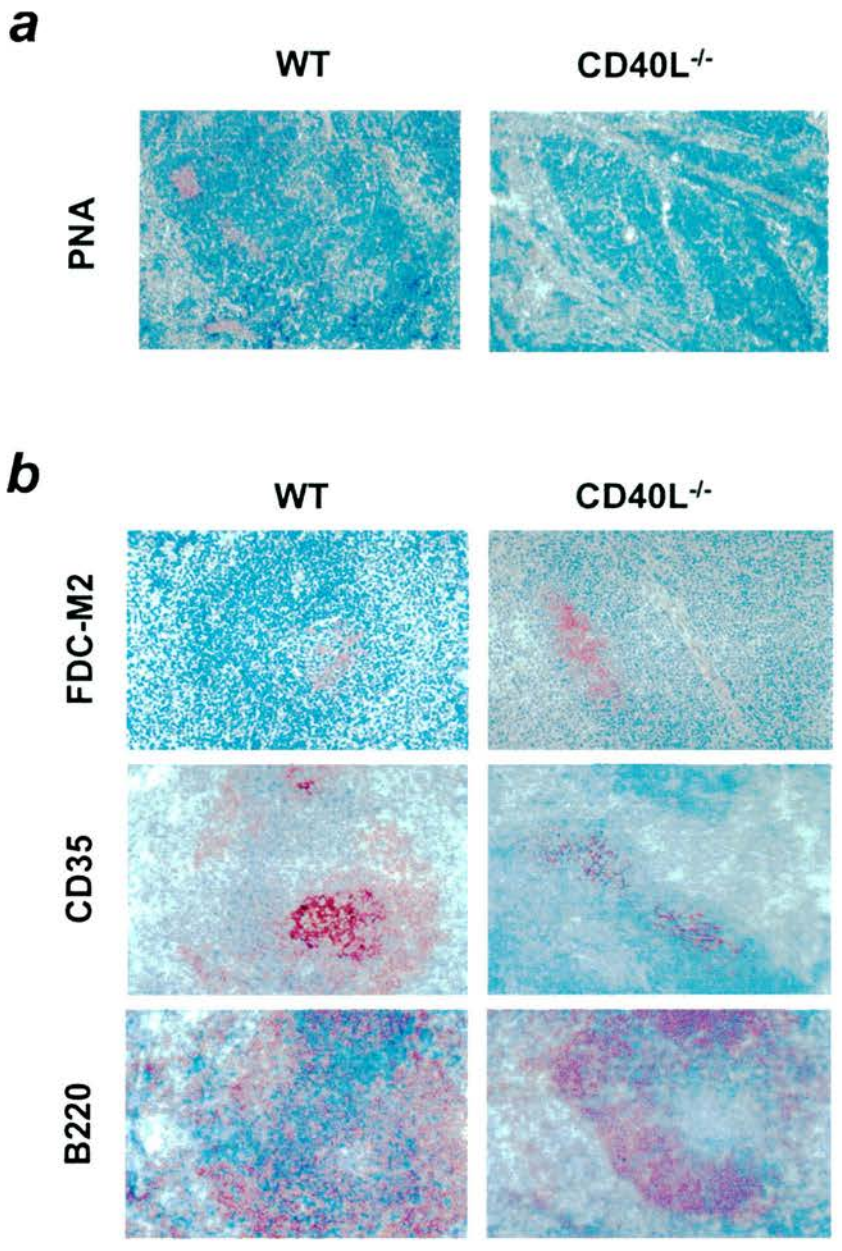


Figure 7.6- Germinal centre architecture in spleens of C57BL/6 wild-type (WT) mice and $CD40L^{-/-}$ mice. (a) Immunohistochemical analysis detected the presence of PNA-positive germinal centre B lymphocytes in spleens from WT mice (red; left hand panel) but not in tissue from $CD40L^{-/-}$ mice (right hand panel). Original magnification, X100. (b) Immunohistochemical analysis of FDC networks in spleen tissue from WT (left hand panels) and $CD40L^{-/-}$ mice (right hand panels). Adjacent frozen sections were stained with FDC-M2 (upper row; red) monoclonal antiserum to detect FDCs, 8C12 monoclonal antiserum to detect CD35 (middle row; red), and B220 monoclonal antiserum to detect B lymphocytes (lower row; red). Original magnification, X200. All sections were counterstained with haematoxylin (blue).

lymphoid tissues of CD40L$^{-/-}$ mice, mature FDC networks surrounded by B-lymphocytes (CD45R/B220 positive cells) were detected (Fig. 7.6b). Expression of the markers FDC-M2 and CD35 by FDCs in spleens of CD40L$^{-/-}$ mice appeared similar to that observed in lymphoid tissues from C57BL/6 wild type mice (Fig. 7.6b). Thus, these data show that lymphoid tissues of CD40L$^{-/-}$ mice contained mature FDC networks and lymphocytes arranged in a manner indistinguishable from those of wild-type mice.

7.3.4 Effect of CD40L deficiency on accumulation of the scrapie agent in lymphoid tissues.

Next the early delivery of the scrapie agent from the skin to the draining lymphoid tissues in the absence of migratory LCs in CD40L$^{-/-}$ mice was studied. ILNs draining the site of inoculation and spleens were taken from two wild-type mice and two CD40L$^{-/-}$ mice 49 days after inoculation with the scrapie agent by skin scarification (1.0% scrapie brain homogenate). The scrapie infectivity titres in pooled (n = 2) tissue homogenates were estimated by bioassay in groups of up to 12 indicator mice. As expected draining ILNs from wild-type mice contained high levels of scrapie infectivity (approximately 5.8 log i.c. ID_{50}/g). In the absence of efficient LC migration from the skin the early accumulation of scrapie infectivity in the draining ILN was not impaired, as high levels of infectivity were also detected in the of ILNs from CD40L$^{-/-}$ mice taken 49 days post inoculation (approximately 5.9 log i.c. ID_{50}/g).

Only trace levels of infectivity (< 2.5 log i.c. ID_{50}/g) were detected in pooled spleen samples from the same wild-type mice and $CD40L^{-/-}$ mice taken 49 days post inoculation. However, at the terminal stage of disease abundant detergent insoluble, relatively proteinase K-resistant accumulations of PrP^{Sc} were detected in spleens from wild-type mice (Fig. 7.7 lanes 2 and 4) and $CD40L^{-/-}$ mice (Fig. 7.7 lanes 6 and 8). Taken together, these data demonstrate that after inoculation via the skin, infectivity is propagated to the draining lymph node via the lymphatics in a LC-independent manner, and subsequently distributed to the spleen, probably via the bloodstream. As only trace levels of infectivity are measured in the spleen at a time when high levels of infectivity are detected in the draining lymph node, these data suggest that the initial transmission from the skin to local lymphoid tissues does not occur via the bloodstream.

7.3.5 Susceptibility of $CD40L^{-/-}$ mice to scrapie infection.

When challenged i.c. with a moderate dose of the scrapie agent (1.0% scrapie-brain homogenate from a terminally scrapie-affected mouse), wild-type mice and $CD40L^{-/-}$ mice developed clinical signs of scrapie with similar incubation periods of 159 to 165 days (Table 7.2). Histopathological analysis of brain tissue from terminally affected wild-type and $CD40L^{-/-}$ mice displayed the characteristic spongiform pathology, disease-specific PrP accumulation and gliosis typical of i.c. inoculation with the ME7 scrapie strain, although microglia activation appeared more pronounced in the brains of $CD40L^{-/-}$ mice (Fig. 7.8). However, the severity and distribution of pathological vacuolation in the brain was not significantly different between mouse strains (Fig. 7.9 a). Thus these data suggest that the CD40L

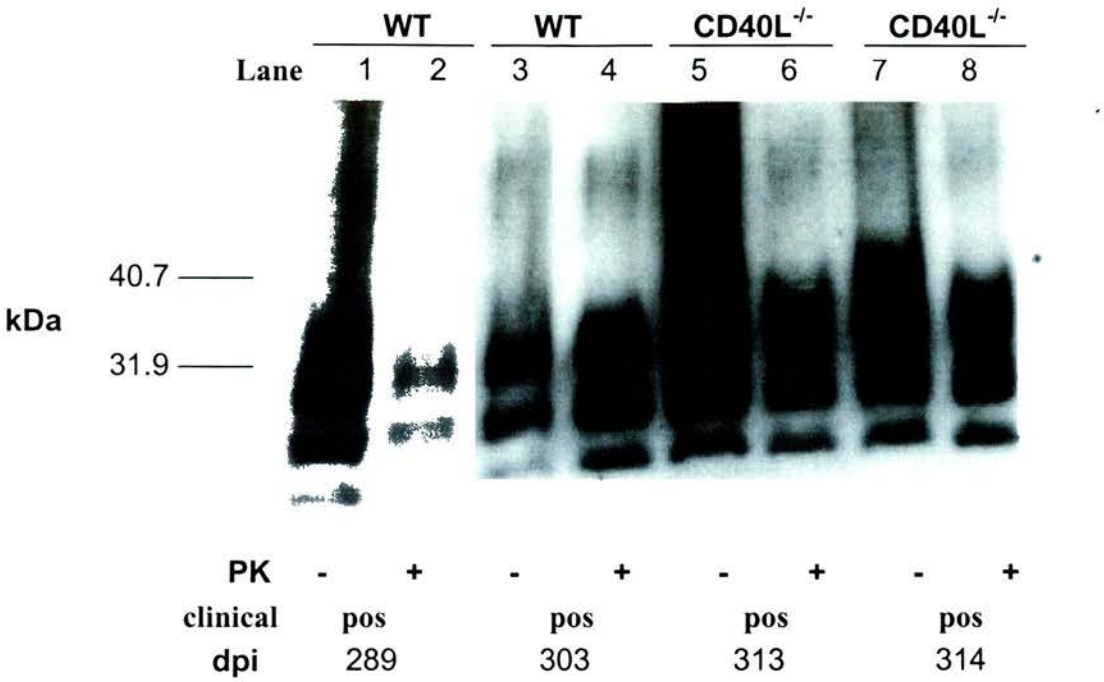


Figure 7.7- PrP^{Sc} accumulation in spleen tissue from terminally scrapie-affected C57BL/6 wild-type (WT) (lanes 2 and 4) and $CD40L^{-/-}$ mice (lanes 6 and 8) inoculated with scrapie by skin scarification. Immunoblots show the accumulation of detergent insoluble, relatively proteinase K (PK) resistant PrP^{Sc}. Treatment of tissues in the presence (+) or absence (-) of PK prior to electrophoresis is indicated. pos., mice that developed clinical signs of scrapie; dpi., days post-inoculation at which the tissues were taken for analysis.

TABLE 7.2- Susceptibility of wild-type (C57BL/6) and CD40L$^{-/-}$ mice to scrapie after inoculation via skin scarification or i.c. injection.

	Skin scarification[a]				i.c. inoculation	
	1% (wt/vol)		0.1% (wt/vol)		1% (wt/vol)	
Mouse Strain	Scrapie incidence	Incubation period[b]	Scrapie incidence	Incubation period	Scrapie incidence	Incubation period
C57BL/6	**8/8**	**344 ± 5**	**8/9**	**355 ± 13, 1X > 504[c]**	**5/5**	**165 ± 1**
CD40L$^{-/-}$	**9/10[d]**	**312 ± 5**	**7/8[e]**	**331 ± 6**	**6/6**	**159 ± 1**

[a], Mice were inoculated with the ME7 scrapie strain by skin scarification of the medial surface of the right thigh.

[b], In days, mean ± S.E.M.

[c], One mouse remained free from the signs of scrapie 504 days post-inoculation at which point the experiment was terminated. No histopathological signs of scrapie were detected in the brain.

[d], One mouse died at 306 days post-inoculation. No histopathological signs of scrapie were detected in the brain.

[e], One mouse died at 359 days post-inoculation. No histopathological signs of scrapie were detected in the brain.

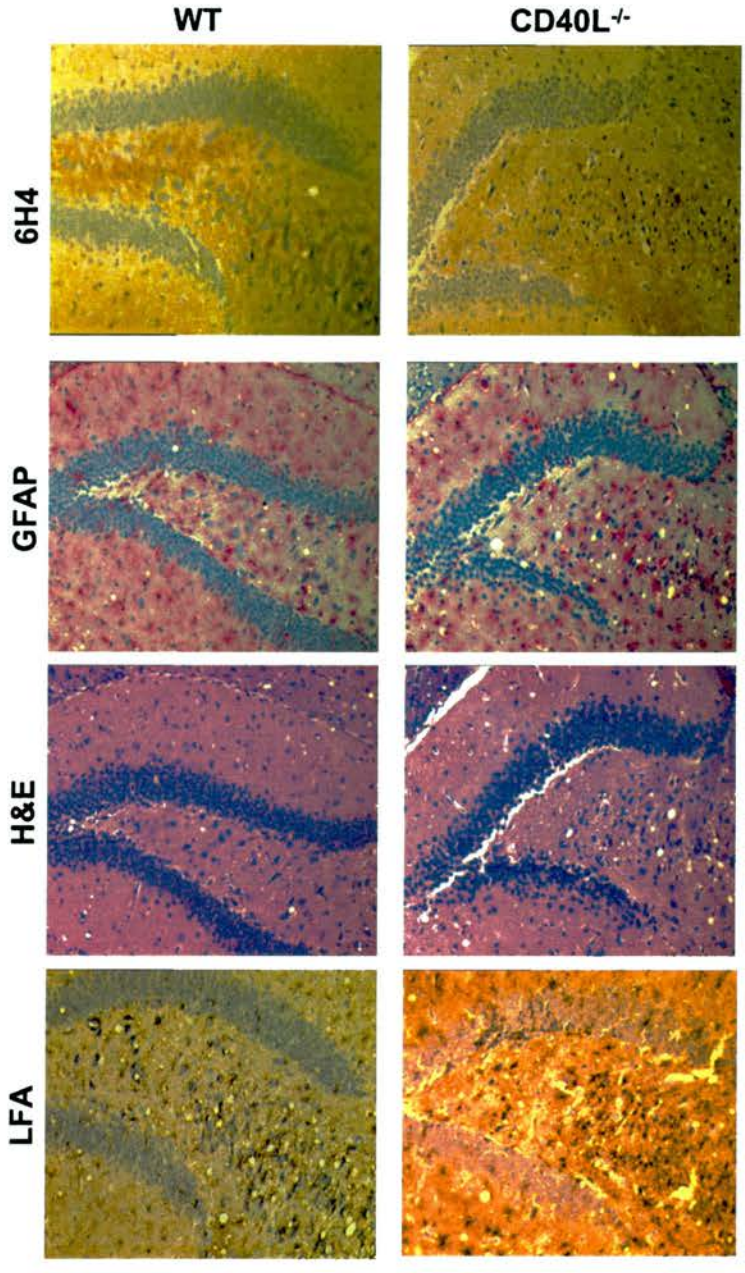


Figure 7.8– Histological analysis of brain tissue from terminally scrapie affected, wild-type (WT) mice (left hand column) and CD40L$^{-/-}$ mice (right hand column) inoculated intracerebrally with scrapie strain ME7. Large PrP accumulations (brown) were detected in the hippocampi of all mice which developed clinical signs of scrapie (top row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP) (red) shows diffuse gliosis in the hippocampi of all terminally scrapie affected mice (second row). Adjacent sections were also stained with hematoxylin and eosin and all displayed extensive vacuolation (third row). Analysis of LEA-binding microglia suggested their activation was more pronounced in the brains of CD40L$^{-/-}$ mice when compared to wild-type mice (bottom row). All sections were counterstained with hematoxylin (blue). Original magnification X200.

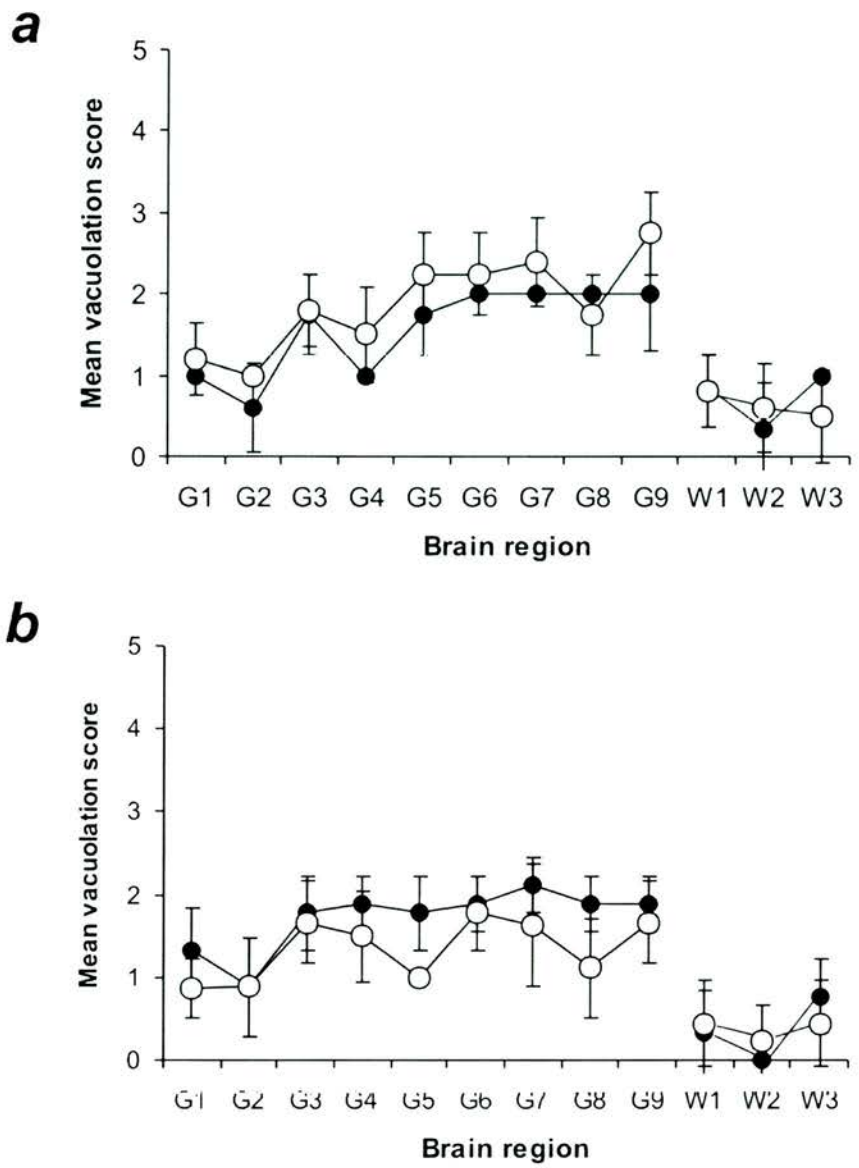


Figure 7.9- Similar pathological targeting in the brains of terminally scrapie-affected $CD40L^{-/-}$ mice and C57BL/6 wild-type mice. Mice were inoculated with the ME7 scrapie strain by i.c. injection (a) or via skin scarification (b). Vacuolation in the brain was scored on a scale of 0-5 in the following grey-matter (G1-G9) and white-matter (W1-W3) areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, Decoction of superior cerebellar peduncles; W3, Cerebral peduncles. Each point represents mean vacuolation score ± S.E.M for groups of 5-9 mice. •, C57BL/6 wild-type mice. ○, $CD40L^{-/-}$ mice.

signalling pathway is not involved in the cerebral pathogenesis following inoculation with the ME7 scrapie strain.

After inoculation with the scrapie agent by skin scarification, $CD40L^{-/-}$ mice developed clinical disease significantly earlier than wild-type mice (Table. 7.2). For example, after inoculation with a moderate dose of the scrapie agent (1.0% brain homogenate), all wild-type mice developed clinical signs of scrapie with a mean incubation period of 344 ± 5 days (n = 8). In contrast, $CD40L^{-/-}$ mice developed clinical scrapie 32 days earlier than the wild-type controls, with a mean incubation period of 312 ± 5 days (P = 0.0003). Characteristic spongiform pathology and disease-specific PrP accumulations and gliosis typical of an infection with the ME7 scrapie strain were detected in the brains of all peripherally inoculated wild-type and $CD40L^{-/-}$ mice (Fig. 7.10). Although the activation status of LEA-binding microglia appeared more pronounced in the brains of $CD40L^{-/-}$ mice when compared to wild-type mice (Fig. 7.10), no significant difference in the severity or distribution of vacuolation in the brain was observed between mouse strains (Fig. 7.9 b). Thus the shortened incubation period in $CD40L^{-/-}$ mice was unlikely to be attributed to an exaggerated neuropathology in these mice when compared to wild-type mice.

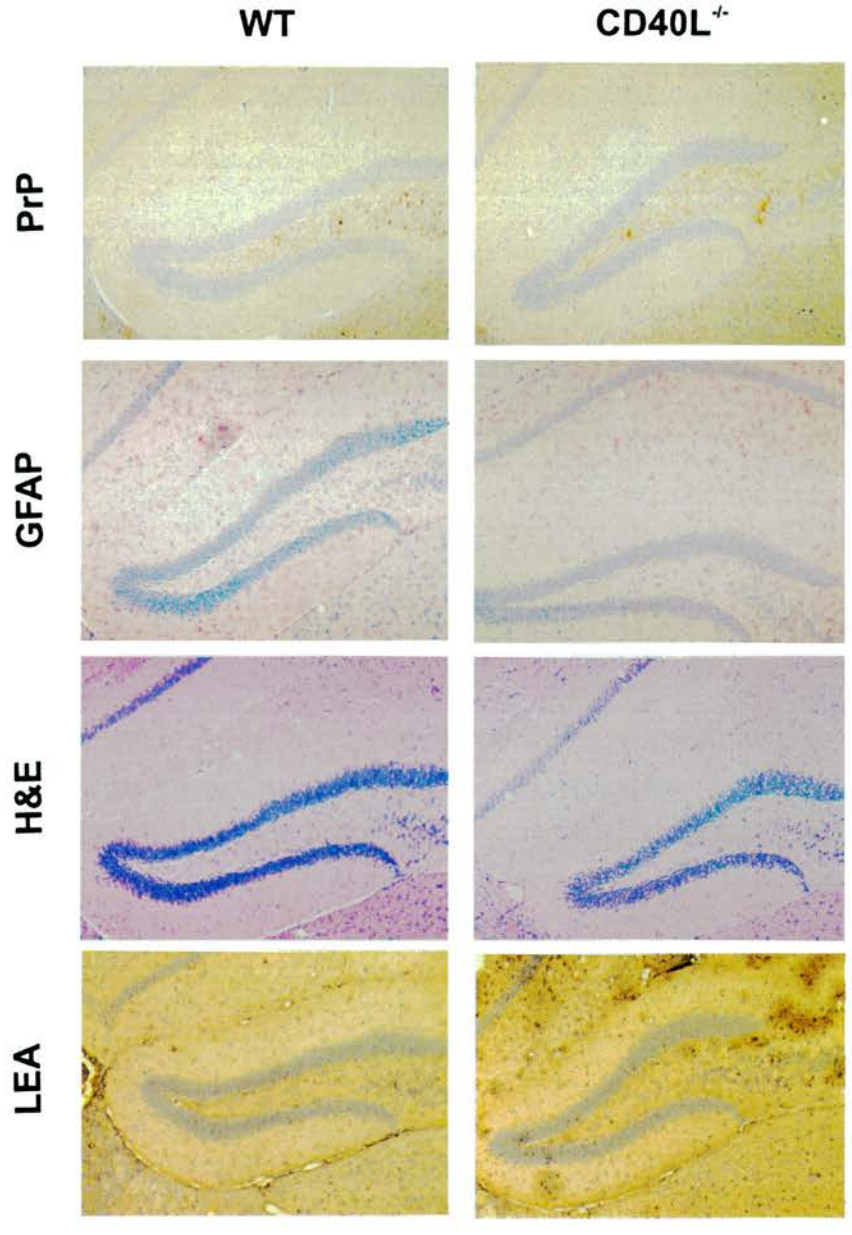


Figure 7.10- Histological analysis of brain tissue from terminally scrapie-affected C57BL/6 wild-type mice (WT; left hand column) and CD40L$^{-/-}$ mice (right hand column) inoculated with scrapie by skin scarification. Large PrP accumulations (brown) were detected in the hippocampi in all mice which developed clinical signs of scrapie (upper row). Immunostaining of adjacent sections for glial fibrillary acidic protein (GFAP) shows high levels of gliosis in the hippocampi of all terminally scrapie affected mice (second row; red). Adjacent sections were also stained with hematoxylin and eosin and all displayed extensive vacuolation (H&E; third row). Analysis of LEA-binding microglia suggested their activation was more pronounced in the brains of CD40L$^{-/-}$ mice when compared to wild-type mice (bottom row). All sections were counterstained with hematoxylin (blue). Original magnification, X100

7.4 Discussion

In order to investigate the potential role of LCs in the transportation of the scrapie agent from the skin to draining lymph nodes (DLNs), mouse models were utilised where the migration of LCs from the skin was impaired (Antonopoulos et al., 2001; Moodycliffe et al., 2000). Pharmacological blockade of LC migration (Antonopoulos et al., 2001) by treatment with an irreversible inhibitor of caspase-1 prior to inoculation with the scrapie agent through the skin did not impair the early accumulation of infectivity in draining lymphoid tissue or delay subsequent neuroinvasion. The migration of LCs from the skin is also impaired in $CD40L^{-/-}$ mice (Moodycliffe et al., 2000). The early accumulation of infectivity in draining lymphoid tissues of these mice was likewise not impaired in the absence of LC migration from the skin. Together, these data suggest that LCs are not involved in the transport of the scrapie agent from the skin to lymphoid tissue. When wild-type mice and $CD40L^{-/-}$ mice were challenged with the scrapie agent directly into the CNS, both strains of mice developed clinical signs of scrapie with similar incubation periods and exhibited similar CNS pathology. In contrast, after inoculation via skin scarification the disease incubation period was shorter in $CD40L^{-/-}$ mice when compared to wild-type mice. The shortened disease incubation period in $CD40L^{-/-}$ mice was unexpected and demonstrates that a CD40L-dependent process involved in impeding scrapie neuroinvasion was disrupted due to CD40L deficiency.

Transport mechanisms of the scrapie agent from the site of exposure to the germinal centres in which they replicate are not known. Migratory bone-marrow derived DCs are a credible candidate transport mechanism as these cells sample antigens in the

periphery and transport them to lymphoid tissues (Banchereau et al., 2000). DCs can also retain some protein antigens in native, non-degraded form (Wykes et al., 1998). Recent research has demonstrated that bone-marrow derived DCs can acquire PrP^{Sc} *in vitro* (Huang et al., 2002; Luhr et al., 2004; Luhr et al., 2002) and that a sub-population of migratory DCs has the potential to transport intestinally injected PrP^{Sc} to mesenteric lymph nodes via the lymph (Huang et al., 2002). Others have shown that the prion protein fragment $PrP_{106-126}$ is a chemoattractant and pro-inflammatory for monocyte-derived dendritic cells (Bacot et al., 2003; Kaneider et al., 2003). Furthermore, studies in this thesis have shown that LCs can acquire scrapie brain homogenate in vitro (Chapter 6). Despite these observations direct demonstration of the involvement of DCs in the initial delivery of TSE agents to lymphoid tissues is lacking. Studies by Oldstone and colleagues (Oldstone et al., 2002) have suggested $CD11c^{+}$ DCs are not involved in peripheral TSE pathogenesis. However, in their study mice were inoculated with high doses of the RML scrapie strain which have been shown consistently to bypass the need for replication in lymphoid tissues in other RML scrapie models (Prinz et al., 2002) and in the ME7 scrapie model (Fraser et al., 1996). Here the potential role of LCs in scrapie pathogenesis is investigated after inoculation with doses of the agent that requires amplification in lymphoid tissues prior to neuroinvasion (Taylor et al., 1996a).

The cytokines interleukin-1β (IL-1β) and tumour necrosis factor (TNF)-α play key roles in regulating the migration of LCs from the epidermis to DLNs (Cumberbatch et al., 1997b; Stoitzner et al., 1999). LC migration from the epidermis is blocked following specific inhibition of either of these cytokines (Cumberbatch et al., 1997b).

IL-1β is synthesized as an inactive 31 kDa precursor which is specifically cleaved to release the active 17 kDa IL-1β molecule by the cysteine protease caspase-1 (Thornberry et al., 1992). In mice deficient in caspase-1 and therefore subsequently deficient in IL-1β LC migration from the skin is impaired (Antonopoulos et al., 2001). Furthermore, treatment of skin with Ac-YVAD-cmk, an irreversible inhibitor of caspase-1 (Garcia Calvo et al., 1998; Thornberry et al., 1994), potently blocks induced LC migration from the skin (Antonopoulos et al., 2001).

Treatment of mice with Ac-YVAD-cmk blocks induced LC migration from the skin by approximately 67% (Antonopoulos et al., 2001). Therefore the possibility exists that the magnitude of impaired LC migration was insufficient to observe a measurable effect on disease pathogenesis. However, this assumption relies on a 67% reduction in LC migration correlating to a direct 67% reduction in delivery of the scrapie agent to the DLNs. Previous studies have shown that only a small subpopulation of intestinal DCs (0.5-5%) are required to transport detectable levels of PrP^{Sc} from the gut lumen to the DLNs (Huang et al., 2002; Huang et al., 2000). Thus, it is likely that the majority of LC sub-populations, if involved in the transportation of the scrapie agent, would be retained within the 67% of LCs unable to migrate from the epidermis, after Ac-YVAD-cmk treatment. After peripheral inoculation it is likely that a portion of the original inoculum is degraded by macrophages (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982) prior to its replication in association with FDCs. This effect is dose dependent: with small doses being more easily destroyed than a higher dose where a greater portion would be retained (Prinz et al., 2002). Therefore, if a 67% reduction

in LC migration did correlate with a 67% reduction in initial delivery of the scrapie agent to the DLNs then the fraction of original inoculum available for degradation would be smaller and hence further reduce the inoculum titre, hence affecting disease pathogenesis. With the above points in mind, it is believed that the effects of Ac-YVAD-cmk treatment on LC migration would be sufficient to cause a measurable effect on scrapie pathogenesis if LCs were involved. However, to further address this issue a $CD40L^{-/-}$ mouse model was also utilised where there is a permanent block in LC migration from the epidermis (Moodycliffe et al., 2000).

CD40-CD40L interactions have also been shown to play an important role in regulating the migration of antigen-bearing LCs to the DLNs as their migration from the epidermis is blocked in $CD40L^{-/-}$ mice (Moodycliffe et al., 2000). This defect in LC migration was associated with defective TNF-α production in the skin (Moodycliffe et al., 2000). Data in this chapter show that blockade of LC migration from the epidermis through treatment with Ac-YVAD-cmk prior to inoculation with the scrapie agent did not effect the early accumulation of infectivity in draining ILNs or delay subsequent neuroinvasion. High levels of scrapie infectivity were also detected within the ILNs of wild-type and $CD40L^{-/-}$ mice 49 days post-inoculation. A time point previously suggested in this thesis (Chapter 3, Fig 3.1) to be the point when infectivity levels peak in the draining ILN. Thus, the early accumulation of the scrapie agent in the draining lymphoid tissue was not impaired in mice with defective LC migration. Therefore the initial transportation of the scrapie agent from the skin to draining lymphoid tissue occurs through a LC-independent mechanism.

Every effort was taken at the time of skin scarification to avoid causing damage to the dermis or drawing blood to prevent the leakage of infectivity into the bloodstream during inoculation. If scrapie infectivity had leached into the bloodstream, a need for LCs in transportation to lymphoid tissues would obviously have been by-passed. Analysis of spleen tissue 49 days post-inoculation failed to detect infectivity in tissues collected from the same wild-type and $CD40L^{-/-}$ mice. These data therefore suggest that the initial transmission of the scrapie agent from the skin to lymphoid tissues does not occur via the bloodstream which would have resulted in a widespread dissemination of infectivity throughout the lymphoid system. Spleen tissue collected for immunoblot analysis from terminally-affected wild-type and $CD40L^{-/-}$ mice detected large accumulations of PrP^{Sc}. Therefore, after inoculation via the skin infectivity is first propagated to the draining lymph node via the lymphatics in a LC-independent manner and subsequently distributed to the spleen, probably via the bloodstream. These data are concurrent with data presented in Chapter 3 and 5 of this thesis.

Mice deficient in CD40L are incapable of mounting T-cell dependent humoral immune responses (Xu et al., 1994). Although mature FDC networks surrounded by B-cells were detected in the spleens of $CD40L^{-/-}$ mice, germinal centres containing PNA-binding B lymphocytes were absent. After inoculation by skin scarification $CD40L^{-/-}$ mice accumulated levels of PrP^{Sc} and infectivity in their lymphoid tissues comparable to wild-type mice, despite an absence of germinal centres. Thus, the absence of PNA-positive germinal centres in $CD40L^{-/-}$ mice does not affect the ability of their lymphoid tissues to accumulate PrP^{Sc} and infectivity after exposure to

the ME7 scrapie strain. These data are consistent with previous studies utilizing interleukin-6 deficient (IL-$6^{-/-}$) mice which also have diminished germinal centres but mature FDC networks in the spleen (Mabbott et al., 2000b). After peripheral inoculation with ME7 scrapie, spleens of IL-$6^{-/-}$ mice accumulated levels of PrP^{Sc} and scrapie infectivity comparable to those in spleens of wild-type mice and maintained these levels for the duration of the disease.

Congruent with the observation that the early accumulation of scrapie infectivity in lymphoid tissues was not impaired in the absence of LC migration, further studies demonstrated that scrapie susceptibility was not reduced in mice with blocked LC migration. When challenged with the scrapie agent by skin scarification, $CD40L^{-/-}$ mice succumbed to clinical disease with a significantly shorter incubation period than wild-type mice (Table. 7.2). The shortened incubation period in $CD40L^{-/-}$ mice was unexpected as a similar affect on scrapie pathogenesis was not observed after inhibition of LC migration by topical treatment with a caspase-1 inhibitor (Table. 7.1). CD40 signaling on neurons has been shown to play a physiological role in promoting neuronal maintenance and survival (Tan et al., 2002). Thus the shortened scrapie incubation period observed in peripherally-inoculated $CD40L^{-/-}$ mice might have been due to an increased susceptibility of CD40L-deficient neurons to TSE-induced neurodegeneration. Following inoculation of wild-type and $CD40L^{-/-}$ mice with the scrapie agent directly into the CNS (i.c. inoculation), all mice developed clinical disease with similar incubation periods. Furthermore, the pathological targeting of the scrapie-induced vacuolation (Fig. 7.8b), disease-specific PrP accumulation, or gliosis (Fig. 7.7) within the brain was not significantly different

between mouse strains, although microglia activation appeared more pronounced in CD40L$^{-/-}$ mice. Despite this apparent increase in microglia activation, the magnitude and distribution of the disease-specific vacuolation in the 12 different brain regions were similar between the CD40L$^{-/-}$ and wild-type mice. These data demonstrate that the CD40-CD40L signaling pathway does not play a critical role in the cerebral pathogenesis of ME7 scrapie. Therefore, the shortened incubation period observed in peripherally inoculated CD40L$^{-/-}$ mice was most likely due to an absence of a CD40L-dependent mechanism in the periphery. These data also suggest that a CD40-CD40L dependent mechanism is involved in impeding scrapie pathogenesis.

This is in contrast to recent data by Burwinkel *et al* (Burwinkel et al., 2004) that suggests that the CD40-CD40L signaling pathway is involved in the development and progression of the disease in the CNS following inoculation with 139A scrapie strain. In their study CD40L$^{-/-}$ mice succumb to disease 40 days earlier than wild-type control mice following i.c. inoculation. Congruent with the findings in our study, no significant differences in the extent of disease-specific PrP accumulations or GFAP-expressing astrocytes were detected in the brains of CD40L$^{-/-}$ and wild-type mice. However their study detected more pronounced microglial activation and disease associated vacuolation in CD40L$^{-/-}$ mice than controls. This is in contrast to this study were the magnitude and distribution of vacuolation in CD40L$^{-/-}$ mice was remarkably similar to wild-type mice following inoculation either directly into the CNS or the skin (Fig. 7.9 a and 7.9 b). In wild-type mice the distribution and magnitude of vacuolation within the brain is significantly different between 139A and ME7 scrapie strains; for example the 139A scrapie strain induces stronger white

matter vacuolation. Therefore taken together these two studies suggest that it is possible the effects of CD40L on scrapie pathogenesis within the brain are strain-dependent.

CD40-CD40L interactions have a diverse range of activities within the immune system and further studies are necessary to determine the precise effect of the blockade of this signalling pathway on peripheral scrapie pathogenesis. Apart from a lack of PNA-binding germinal centres (Xu et al., 1994), there was little evidence of disrupted lymphoid architecture in lymphoid tissues of $CD40L^{-/-}$ mice. Thus the reduced incubation period was unlikely to be due to affects on lymphoid architecture, such as the repositioning of splenic FDCs in close association with peripheral nerves as recently shown in mice deficient in the CXCR5 chemokine receptor (Prinz et al., 2003b).

CD40-CD40L interactions provide important signals for optimal macrophage activation and CD40L-deficiency increases susceptibility to certain intracellular pathogens (Campbell et al., 1996; Marriott et al., 1999; Soong et al., 1996). For example, macrophages from Leishmania-infected $CD40L^{-/-}$ mice contain high parasite burdens and are unable to effectively control the parasitemia (Soong et al., 1996). As studies suggest that macrophages may sequester and destroy scrapie infectivity (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982), it is reasonable to speculate that in the absence of CD40-CD40L signalling in $CD40L^{-/-}$ mice the clearance of scrapie infectivity by cells such as macrophages is likewise inhibited. For example, studies from Prinz and colleagues (Prinz et al.,

2002) demonstrate that in the absence of TNF receptor 1-signaling, replication of the scrapie agent can occur in macrophages.

Following uptake by DCs, antigens rapidly enter the lysosomal compartment where they are broken down into peptides for presentation to lymphocytes in association with MHC class II (Banchereau et al., 2000). A recent study suggests that DCs may handle TSE agents in a similar manner, as bone-marrow-derived DCs are able to process and degrade PrP^{Sc} following *in vitro* exposure (Luhr et al., 2004; Luhr et al., 2002). Similarly, studies in this thesis have shown that LC-like cells are capable of degrading PrP^{Sc} and scrapie infectivity. Thus, highly efficient antigen processing LCs might be impeded in processing of the scrapie agent in CD40L-deficient mice. Inhibition of CD40L-dependent clearance mechanisms might allow infectivity to accumulate earlier in draining lymphoid tissue shortening the disease incubation period. Treatments that modulate this CD40L-dependent protective mechanism might provide an effective therapeutic strategy for early intervention in TSE pathogenesis.

Data presented here suggest that LCs are not involved in the active transportation of the scrapie agent from the skin to lymphoid tissues. However these data do suggest that a CD40-CD40L dependent mechanism is involved in impeding scrapie neuroinvasion. The precise mechanism by which scrapie infectivity is transported from the skin to lymphoid tissues remains to be identified. Experiments in this chapter only addressed the role of active LC migration in the transportation of scrapie, it is possible that LCs may transport scrapie to the DLN in a steady-state.

Furthermore, it is possible that scrapie infectivity is transported in a cell-free manner. Soon after entering the host antigens are rapidly opsonised by complement components. Recent research has shown that complement components play an important role in the localization of the scrapie agent to lymphoid tissues (Ishii et al., 1984; Klein et al., 2001; Kovacs et al., 2004; Lotscher et al., 2003; Mabbott et al., 2001) suggesting the scrapie agent might be delivered to lymphoid tissues as cell-free complement-bound complexes. Understanding the earliest peripheral mechanisms involved in the transportation or degradation of the scrapie agent will be important in the development of therapeutic strategies to combat these fatal neurodegerative diseases.

8

General Discussion/Future Work

8.1 Infection of the skin with TSE agents

Studies in this thesis have demonstrated that following transmission via the skin low levels of scrapie infectivity persist in the skin for up to 7 days after inoculation (Chapter 3). It is not known which cells within the skin the scrapie agent associates with during this time period. Previous studies have shown that both keratinocytes and Langerhans cells (LCs) within the epidermal layers of the skin express the host cellular form of the prion protein PrP^C (Pammer and Tschachler, 2002; Pammer et al., 1998; Sugaya et al., 2002). This raised speculation that these cell types might be the first cellular targets for TSE agent replication following exposure via the skin. However, studies reported here have demonstrated that scrapie infectivity does not replicate in the skin early in the disease incubation period (Chapter 3). During the clinical phase of scrapie in sheep the animals develop a chronic itch of the skin (McGowan, 1922; Scott, 1993), suggesting that it is possible that scrapie infectivity might return to the skin later in the incubation period as the disease becomes more systemic. For example, infectivity is detectable in the skin of greater kudu affected with BSE (Cunningham et al., 2004). However, it is also conceivable that the itching displayed by scrapie-affected sheep is due to the degeneration of sensory neurons within the central nervous system (CNS). Taken together these data suggest that it is unlikely that scrapie infectivity is transmitted via abraded skin during the early stage of the disease, but this route of transmission might occur during the clinical phase.

Keratinocytes in the epidermis of the skin are continually shed from the skin and are completely renewed within approximately 28 days (Williams, 1995). As the level of scrapie infectivity present declines to trace levels by 7 days after inoculation, it is

likely that much of the original infectivity is shed from the skin. It is also possible that in a clinically affected animal the shedding of TSE-infected cells from the epidermis could result in environmental contamination and subsequent spread of infection. For example, studies have demonstrated that animals can acquire TSE infection through exposure to other TSE-infected animals(Brotherston et al., 1968; Haralambiev et al., 1973; Miller et al., 2004) or exposure to pastures previously inhabited by TSE- affected animals(Greig, 1940; Miller et al., 2004; Palsson, 1979). As PrP^{Sc} is relatively resistant to degradation (Brown and Gajdusek, 1991; Somerville et al., 2002) it is possible that TSE-infected epithelial cells could contaminate the natural environment. For example, a scrapie-affected sheep scratching against a fence post might leave scrapie-infected epidermal cells on the post, which then might be transferred to another animal scratching against the same post.

Alternatively, the natural transmission of TSE agents via the skin might occur via ectoparasites (Lupi, 2003). Infestation by ecoparasites is common occurrence in sheep as well as other wild and domestic animals. Studies have demonstrated that flies (*Sarcophaga carnaria*) (Post et al., 1999) and mites (*Lepidoglyphus destructor*, *Acarus farris*) (Wisniewski et al., 1996) can acquire infectivity when exposed to TSE-affected brains, suggesting that ectoparasites might function as reservoirs or vectors for the transmission of TSE agents in the natural environment.

8.2 Transport of TSE agents from the skin

8.2.1 Langerhans cells

Studies in this thesis have shown that scrapie infectivity first accumulates to high levels in the draining lymph node (DLN) following transmission via the skin (Chapter 3). However, it is not known how TSE agents are transported from the site of inoculation to the DLN. Previous research has shown that a sub-population of dendritic cells (DCs) are capable of transporting PrP^{Sc} from the gut lumen to the mesenteric lymph nodes (Huang et al., 2002). LCs are a subset of DCs that reside in the epidermis and migrate to the DLN following antigen encounter (Banchereau et al., 2000). In this thesis it was hypothesised that LCs might actively transport the scrapie agent from the skin to the DLN (Chapter 7). In these studies mouse models were utilised in which active LC migration was inhibited either due to CD40 ligand-deficiency ($CD40L^{-/-}$ mice) (Moodycliffe et al., 2000), or following caspase-1 inhibition (Antonopoulos et al., 2001) (Chapter 7). These studies demonstrated that the transport of the scrapie agent from the skin to the DLN was not impaired in the absence of active LC migration (Chapter 7). However, recent studies have demonstrated that LCs can also migrate from the epidermis in a steady-state without becoming immunologically activated (Hemmi et al., 2001; Yoshino et al., 2003). These studies suggested that steady-state migration might be a pathway employed following encounter with self-antigen, where high levels of pro-inflammatory cytokines are not produced. Thus, this would prevent the up-regulation of immunostimulatory molecules, which could induce autoreactivity. As PrP^{Sc} is likely to be considered a self-antigen due to its similarity to the host protein PrP^{C} (Stahl et al., 1993), it is possible that LCs might transport TSE agents via a steady-state

migratory pathway. This hypothesis is supported by studies in this thesis which demonstrate that LCs do not become immunologically activated following *in vitro* exposure to the scrapie agent (Chapter 6).

Alternatively, LCs might have a similar role in TSE pathogenesis to that of macrophages, which have been suggested to play a dual role in scrapie pathogenesis (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982; Prinz et al., 2002). Macrophages are considered to degrade the TSE agent (Beringue et al., 2000b; Carp and Callahan, 1981; Carp and Callahan, 1982), but in some circumstances may also accumulate PrP^{Sc}, especially following exposure to high doses of inoculum (Prinz et al., 2002). Experiments in this thesis demonstrate that that LCs can partially degrade the scrapie agent, however data also shows that these cells can retain PrP^{Sc} following LPS-stimulation (Chapter 6). Thus, the role of LCs in TSE pathogenesis might be dictated by the microbiological and immune status of the host. Previous studies in mice have demonstrated that treatment with LPS prior to intra-peritoneal inoculation with the scrapie agent increased the efficiency of scrapie infection (Kimberlin and Walker, 1990). The mechanism by which LPS increased the efficiency of infection was not identified. It has been suggested that LPS-stimulation might reduce the amount of infectivity taken up by phagocytic cells (Kimberlin and Walker, 1990), possibly by reducing their responsiveness to a second antigen (Steinman, 1991). These data suggest that transmission of TSE agents via the skin in the naturally occurring disease in the field might be more efficient than experimental transmissions due to the increased exposure to antigens in the natural environment.

8.2.2 Peripheral nerves

The skin is highly innervated containing predominately-unmyelinated C-fibre nerves that are present within both the dermis and epidermis (Williams, 1995). The nerves at the site of inoculation (medial surface of the thigh) in this study belong to the obturator nerve, which arises from the second to fourth lumbar ventral rami of the spinal column (Berry et al., 1995). Previous studies have shown that demylinated nerves are more permissive to scrapie infection (Kimberlin et al., 1983a). It is therefore conceivable that following skin scarification the TSE agent could be directly transported from the skin by peripheral nerves to the spinal column and then to the brain. However, studies by Taylor *et al* (Taylor et al., 1996a), and studies reported in this thesis (Chapter 4), have demonstrated that highly immunodeficient SCID mice are refractory to scrapie infection following transmission via the skin. These data suggest that the scrapie agent is unable to reach the CNS directly via peripheral nerves in the skin but requires an amplification phase within the lymphoid tissues prior to neuroinvasion. The nerves in the skin at the site of inoculation might contain TSE infectivity. However, it is possible that the amount of agent available for uptake by nerves within the skin is insufficient to induce the clinical disease within the lifespan of the host. Thus, an obligatory accumulation phase within lymphoid tissues (Chapter 4 and 5) is required to generate the threshold levels of infectivity required to induce clinical disease. Alternatively, the nerves within the skin might not be permissive for infection and a systemic spread of infectivity is required to access the nerves that are permissive to infection.

.2.3 Dermal dendritic cells

An alternative TSE transport mechanism might be via dermal dendritic cells (DDCs) within the dermis of the skin (Chapter 1; Fig. 1.1.) which provide a LC-independent antigen-presenting pathway (Kurimoto et al., 1994; Streilein, 1989). To determine the role of all DCs in TSE agent transport, a DC-deficient mouse model could be utilised. CD11c is expressed by all murine DC subsets except LC and DDC which only express this surface marker at low levels prior to maturation (Kimber et al., 1999; Larregina et al., 2001; Stoitzner et al., 1999). In CD11c-DTR transgenic mice the diphtheria toxin receptor (DTR) is expressed under the control of the CD11c promoter (Brocker et al., 1997). Murine cells unlike primate cells are insensitive to diphtheria toxin. Therefore, injection of CD11c-DTR transgenic mice with diphtheria toxin temporarily depletes those cells only expressing the DTR (i.e. DCs) (Jung et al., 2002). As skin derived DCs do not express high levels of CD11c this mouse model would not be appropriate for studying the role of skin derived DCs in TSE pathogenesis. However, alternative transgenic mouse models could be generated in which DTR was expressed under the control of the Langerin promoter (Valladeau et al., 2002) or the CD205 promoter (Figdor et al., 2002) to investigate a role for LCs, or the CD1d promoter (Gerlini et al., 2001) to investigate the role of DDCs in the transport of TSE agents.

8.2.4 Cell-free

Alternatively, TSE agents might also be transported from the skin to the DLN via cell free mechanisms. Soon after entering a host some antigens are rapidly bound by complement components (Nielsen et al., 2000; Song et al., 2000). Studies have

shown that after peripheral exposure to antigen immune complexes rapidly localize within the subcapsular sinus of the DLN before localisation to FDCs which express complement receptors CR1, CR2 and CR3 (Kamperdijk et al., 1987; Van De Berg et al., 1995). It is therefore conceivable that the scrapie agent is bound by complement upon entering the host and transported to FDCs via this cell-free mechanism. Studies have already suggested that complement and cellular complement receptors may play a role in the localization and retention of TSE agents by FDCs (Klein et al., 2001; Lotscher et al., 2003; Mabbott et al., 2001). These studies have demonstrated that the absence of early complement components such as C1q and C3 the disease incubation period is significantly extended (Klein et al., 2001; Lotscher et al., 2003; Mabbott et al., 2001). To investigate whether TSE agents are transported from the skin to the DLN in a cell-free or a cell-associated manner the afferent lymphatics draining the site of inoculation could be cannulated and cells and lymph draining the site of inoculation collected. However, this would be technically difficult to perform in mice due to the small size of lymphatic vessels. However, this technique has already been performed in rat models intra-intestinally infected with the TSE agent (Huang et al., 2000).

8.3 Accumulation of TSE agents in lymphoid tissues

Following peripheral exposure, high levels of TSE infectivity and PrP^{Sc} usually accumulate in lymphoid tissues prior to the dissemination of infection to the CNS (Brown et al., 1999; Eklund et al., 1967; Farquhar et al., 1994; Kimberlin and Walker, 1979; Mabbott et al., 2000b). At present the targeting of the LRS prior to neuroinvasion has been demonstrated in vCJD patients (Hilton et al., 1998), CWD in

deer and elk (Sigurdson et al., 1999), sheep with natural scrapie (van Keulen et al., 1996) and rodents experimentally inoculated with scrapie (McBride et al., 1992). Studies of experimental rodent models inoculated intra-peritoneally or orally with the scrapie agent have shown that mature PrP^{C}-expressing FDCs are critical for the replication and accumulation of TSE agents within lymphoid tissues and its subsequent neuroinvasion (Brown et al., 1999; Klein et al., 1998; Mabbott et al., 2000a; Mabbott et al., 2002; Mabbott et al., 2000b; Mabbott et al., 2003; Montrasio et al., 2000). PrP^{Sc} accumulation in association with FDCs has also been detected in the lymphoid tissues of patients with vCJD (Hilton et al., 1998), sheep with natural scrapie (Andreoletti et al., 2000; Heggebo et al., 2002; Hermann et al., 2003) and deer with CWD (Sigurdson et al., 2002). Similarly, studies in this thesis have demonstrated that following exposure to the scrapie agent via scarified skin infectivity accumulates first in the draining ILN to high levels prior to targeting the non-draining ILN and the spleen (Chapter 3). Furthermore, PrP^{C}-expressing FDCs are critical for the accumulation of the scrapie agent within lymphoid tissues and subsequent neuroinvasion following skin scarification (Chapter 4 and 5). However, it is not known how TSE agents are transported from the DLN to other lymphoid tissues. Studies have previously demonstrated that it is possible to transmit TSE infectivity via blood (Houston et al., 2000; Hunter, 2003; Hunter and Houston, 2002; Llewelyn et al., 2004; Pincock, 2004). Data presented in this thesis suggests that the scrapie agent might be transported from the draining ILN to other lymphoid tissues via the efferent lymphatic into the blood (Chapter 3, 5, 7), as similar levels of scrapie infectivity are first detected within the non-draining ILN and spleen at the same time point after inoculation.

The importance of the spleen for the accumulation and replication of TSE agents following peripheral challenge has been demonstrated by genetic asplenia and splenectomy of rodents either prior to or shortly after TSE inoculation (Clarke and Haig, 1971; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978). In the absence of the spleen around the time of inoculation the disease incubation period is significantly prolonged (Clarke and Haig, 1971; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978). However, the presence of the spleen is not obligate for establishing scrapie infection, as splenectomy does not affect disease susceptibility (Clarke and Haig, 1971; Fraser and Dickinson, 1970; Fraser and Dickinson, 1978). Furthermore, splenectomy of mice either before or after subcutaneous inoculation with TSE agents has no affect on the disease incubation period (Fraser et al., 1992; Kimberlin and Walker, 1989a). This suggests that other lymphoid tissues accumulate TSE agents in the absence of the spleen. Similarly, data presented in this thesis suggests that the spleen is not critical for the accumulation and subsequent neuroinvasion of the scrapie agent following transmission via the skin (Chapters 3, 5 and 7). Treatments which temporarily dedifferentiate FDCs, block the early accumulation of PrP^{Sc} and infectivity in lymphoid tissues and significantly delay neuroinvasion when treated prior to peripheral inoculation with TSE agents (Mabbott et al., 2000a; Mabbott et al., 2002; Mabbott et al., 2000b; Mabbott et al., 2003; Montrasio et al., 2000; Prinz et al., 2002). However, these treatments have no effect on TSE pathogenesis once infection is established within the peripheral nervous system (Mabbott et al., 2000a; Mabbott et al., 2003; Montrasio et al., 2000). In this study, temporary dedifferentiation of FDCs within the first two weeks after inoculation with the scrapie agent via the skin delays neuroinvasion (Chapter 5).

However, the lack of any observable effect of treatment on disease pathogenesis when given 42 days after inoculation via the skin (Chapter 5) suggesting that neuroinvasion had occurred by this time. Furthermore, these data suggest that neuroinvasion might have occurred directly from the draining ILN, which contained high levels of PrP^{Sc} at 42 days after inoculation, whereas the spleen and the non-draining lymph node contained only limited levels (Chapter 5).

8.4 How do TSE agents reach follicular dendritic cells?

Once TSE agents reach lymphoid tissues after exposure by skin scarification it is not known how they are transported within lymphoid tissues to FDC networks. If skin derived DCs were responsible for the transportation of TSE agents from the skin to the DLN, then these cell populations would most likely migrate to the paracortex which is anatomically distinct from the outercortex where FDCs reside (von Andrian and Mempel, 2003).

Studies have demonstrated that some migratory DCs containing processed antigen are phagocytosed by resident DCs within lymph nodes (Inaba et al., 1998). Thus, it is possible that the TSE agents might be transferred from skin derived DCs within the paracortical region to other sub-populations of DCs, such as germinal centre dendritic cells (GCDCs) found within the FDC containing outercortex, (Grouard et al., 1996). As GCDCs express both complement and Fc-receptors (Grouard et al., 1996), these cells would also have the potential to acquire complement bound TSE agents.

Studies have shown that a population of skin-derived DCs that express CXCR5 respond to B-lymphocyte chemoattractant (CXCL13) stimulation, allowing them to directly migrate to germinal centres within lymph nodes (Saeki et al., 2000). As DCs can directly transfer native antigen to B-lymphocytes (Batista et al., 2001; Wykes et al., 1998), it is possible that skin-derived DCs could also transfer TSE agents directly to B-lymphocytes within germinal centres (Saeki et al., 2000; Wykes et al., 1998; Yu et al., 2002). However, these cells would have to retain TSE agents in their native form. Although, studies described in this thesis have demonstrated that LCs have the potential to degrade both scrapie infectivity and PrP^{Sc} however this was not evident until at least 48 hours post-exposure (Chapter 6). It is likely that this time would be sufficient for LCs to migrate to the DLN and deliver TSE agents to B-lymphocytes in native form (Austyn et al., 1988). In order to determine whether LCs could transfer the TSE agent directly to other cell populations LCs could be exposed to fluorescently labelled PrP^{Sc} and co-cultured with other cell populations such as B-lymphocytes and FDCs. The transfer of PrP^{Sc} could be determined by FACS analysis of the recipient cell populations.

In addition, the scrapie agent could be disseminated into the microenvironment of the DLN following apoptosis of skin derived DCs. Between 2-3 days after arrival at the DLN, skin derived DCs undergo apoptosis following interactions with T-lymphocytes (Ingulli et al., 1997; Kawamura et al., 1998), releasing apoptotic bodies into the microenvironment of the DLN which are then cleared by phagocytic cells(Jacobson and McCarthy, 2002). Therefore, skin derived DCs infected with TSE agents might transfer them to the phagocytic cells via apoptotic bodies

(Jacobson and McCarthy, 2002). Studies in this thesis have shown that the partial degradation of the scrapie agent by LCs occurs over a 96 hour time period (Chapter 6). *In vivo* it is likely that LCs would have undergone apoptosis prior to degradation of the agent. In order to investigate whether LCs infected with TSE agents undergo apoptosis *in vivo* and release their contents, a reporter transgene could be inserted into a LC cell line and the cells exposed to fluorescently labelled PrP^{Sc}. The PrP^{Sc} containing LCs could then be injected into syngeneic mice and DLNs collected post-inoculation. The presence of functional LCs could be determined by the expression of the reporter gene which would be undetectable following apoptosis. The cellular dissemination of PrP^{Sc} could be determined by FACS analysis or confocal microscopy.

Exosomes are secreted intralumenal contents of multivesicular bodies which are released by many cell types including DCs (Zitvogel et al., 1998) and B-lymphocytes (Raposo et al., 1996). Recent research has demonstrated that exosomes released from PrP^{Sc} neuroglial infected cell lines are capable of transferring PrP^{Sc} both *in vitro* and *in vivo* (Fevrier et al., 2004). These data suggest that TSE agents might be released from skin derived DCs via exosomes which could then fuse with the cell surface of other local cell populations.

TSE agents might also be disseminated within the DLN by a cell-free mechanism. Following peripheral exposure lymph-borne antigens initially drain to the subcapsular sinus of the DLN. Antigens are then transported either directly into the medullary sinuses and leave the DLN via the efferent lymph vessel, or they drain into

a reticular conduit system gaining access to the follicles (von Andrian and Mempel, 2003). Lymph-borne molecules can only drain via the conduit system if they are taken up by resident phagocytic cells or if they are of a molecular weight of less than approximately 60-kDa (Nolte et al., 2003). Studies have shown that a 60-kDa PrP molecule derived from TSE-affected brain is most likely a PrP dimer (Priola et al., 1995). These data suggest that if PrP^{Sc} arrived at the DLN as cell free dimers or aggregates greater than 60 kDa then their size would be to large to permit drainage via the conduit system (Nolte et al., 2003).

8.5 Neuroinvasion by TSE agents

Several studies have demonstrated that TSE agents gain access to the CNS via the neural pathways and ganglia of the peripheral nervous system (Beekes and McBride, 2000; Beekes et al., 1998; Glatzel and Aguzzi, 2000; Glatzel et al., 2001; McBride and Beekes, 1999; McBride et al., 2001). For example, after oral inoculation of hamsters with the 263K scrapie strain, neuroinvasion from the gut occurs via the vagus and splanchnic nerves (Beekes and McBride, 2000; Beekes et al., 1998; McBride et al., 2001). The route of neuroinvasion following inoculation via the skin is not known. The vagus nerve does not constitute the major innervation nervous pathway within the ILNs or the spleen (Felten and Felten, 1991; Mignini et al., 2003) tissues in which scrapie infectivity is first detected following inoculation via the skin. However, studies have shown that after intra-peritoneal inoculation with TSE agents sympathetic nerves within the spleen are important for subsequent neuroinvasion of the agent (Glatzel et al., 2001).

Little is known of the innervations of ILNs in mice. A number of studies have demonstrated that the cervical, mesenteric and popliteal lymph nodes of mice are innervated by both sympathetic and sensory nerve fibres from the lumbar region of the spinal column (Berry et al., 1995; Felten and Felten, 1991; Mignini et al., 2003). These nerves innervate the paracortical, cortical and medullar regions of the lymph node (Felten and Felten, 1991; Mignini et al., 2003). However, the presence of these fibres within the germinal centres has not yet been identified (Mignini et al., 2003). In comparison, approximately 98% of nerve fibres within the spleen are sympathetic (Mignini et al., 2003) and are derived primarily from the superior mesenteric-coeliac ganglion which connects to the thoracic region of the spinal column (Berry et al., 1995; Mignini et al., 2003). Nerve fibres within the spleen can be detected in the periarterial lymphatic sheath, marginal zone, sinuses and the parafollicular zone with the occasional fibre supplying the follicles (Mignini et al., 2003).

At present it is not known how TSE agents are transported from FDCs to the nerves within lymphoid tissues. However, artificial movement of FDCs closer to peripheral nerves, by disruption of chemokine gradients responsible for the localisation of FDCs in lymphoid tissues (Endres et al., 1999; Force et al., 1995; Futterer et al., 1998), enhanced the neuroinvasion of the scrapie agent (Prinz et al., 2003b). This suggests that TSE agent transport to peripheral nerves could occur directly from FDCs. However, it is also possible that TSE agents might be transported to nerves by mobile cells, such as the DCs (Aucouturier et al., 2001). As the lymphoid follicles in the spleen appear to be more innervated than those of the ILN and the nerves from the spleen enter the spinal column at a point closer to the brain than that

observed for the ILNs (Cano et al., 2001; Felten and Felten, 1991; Mignini et al., 2003), it would be reasonable to assume that neuroinvasion would occur faster from the spleen than the ILNs following skin scarification. However, data presented in this thesis suggests that neuroinvasion is most likely to occur first from the draining ILN (Chapter 5). Although, this does not exclude the possibility that neuroinvasion also occurs from the spleen at a later time point. Further studies are needed to determine the precise neuroanatomical pathway by which TSE agents spread to the CNS following inoculation via the skin.

8.6 Potential opportunities for therapeutic intervention

Once TSE agents spread to the CNS the neurodegeneration they cause is likely to be irreversible. Therefore, the development of therapeutic strategies which block the neuroinvasion of TSE agents are more likely to be effective at preventing clinical disease or extending the disease incubation period than those which target the disease within the CNS.

Blockade of the lymphotoxin-β receptor (LTβR) signalling pathway by treatment with LTβR-Ig, results in the temporary dedifferentiation of FDCs within 3 days of treatment for approximately 28 days (Mackay and Browning, 1998). Previous studies have shown that treatment with LTβR-Ig before intra-peritoneal or oral inoculation with the scrapie agent reduces disease susceptibility and extends survival time (Mabbott et al., 2000a; Mabbott et al., 2003; Montrasio et al., 2000). Data in this thesis has shown that treatment with LTβR-Ig likewise extends survival time when given up to 14 days after exposure to the scrapie agent via scarified skin

(Chapter 5). However, FDC dedifferentiation at 42 days after inoculation did not affect neuroinvasion suggesting that the scrapie agent had spread from FDCs to peripheral nerves between 14 - 42 days after inoculation (Chapter 5). These observations indicate that the time period in which FDCs can be manipulated to delay TSE pathogenesis following inoculation via the skin is short.

DCs are the most powerful professional antigen presenting cells within the body (Banchereau et al., 2000; Banchereau et al., 2001; Banchereau and Steinman, 1998). Inaba and co-workers were the first to demonstrate that injection of DCs previously exposed to antigen *in vitro* could generate an immune response *in vivo* (Inaba et al., 1990). Since this initial study DCs have been considered for use as vectors for immunotherapy against cancer (Paczesny et al., 2003) and viral diseases (Ludewig, 2003). TSE infections do not appear to induce humoral (Chandler, 1959; Clarke and Haig, 1966; Marsh et al., 1970; Pattison et al., 1964; Porter et al., 1973) or cellular (Kingsbury et al., 1981) immune responses. Studies in this thesis have shown that LCs can acquire PrP^{Sc} but do not alter their expression of the cellular markers associated with an immune response, suggesting that LCs respond to PrP^{Sc} in a toleragenic manner. Recent research has identified an epitope within the PrP molecule which is displayed specifically by PrP^{Sc} (Paramithiotis et al., 2003) thus, it is possible that DCs could be primed *in vitro* to recognise this epitope and be injected *in vivo* to generate an immune response specific to TSE agents. In conclusion, data in this thesis has demonstrated how understanding the earliest stages of TSE diseases might aid in the development of therapeutic strategies.

8.7 Conclusion

Collectively data presented here demonstrates that scrapie infectivity administered via the skin is transported to the DLN where it accumulates to high levels prior to dissemination to other lymphoid tissues, most likely via the blood (Chapter 3). The mechanism by which the scrapie agent is transported from the skin to the DLN is unknown as the early accumulation of the agent within the DLN and its subsequent neuroinvasion was not impaired in mice with blocked LC migration (Chapter 7). Thus, LCs are unlikely to be involved in the active transportation of TSE agents from the skin. Once the scrapie agent has reached lymphoid tissues, PrP^{C}-expressing FDCs are critical for the accumulation of scrapie infectivity within these tissues (Chapter 4). In the temporary absence of FDCs disease susceptibility is reduced and/or the survival time increased (Chapter 5). Subsequent neuroinvasion from lymphoid tissues appeared to occur between 14 and 42 days post-inoculation (Chapter 5). *In vitro* studies have demonstrated that LCs acquire and degrade the scrapie agent in a non-activated state (Chapter 6). Furthermore, this ability is lost in the presence of LPS, suggesting that LCs could be involved in either the degradation or accumulation of the scrapie agent depending on the microbiological and immunological status of the skin. Although studies presented here show that LCs are unlikely to be involved in the transport of the scrapie agent, further studies are required to determine how the scrapie agent is transported from the site of inoculation to the DLN. Understanding the earliest stages of TSE pathogenesis after inoculation via the skin will aid in the development of therapeutic strategies and help to determine the risk of natural TSE transmission via the skin.

APPENDIX 1: PrP Epitopes

Antiserum	Position in mouse PrP amino acid sequence	Mouse antigenic sequence
1B3	14-36 83-102 119-139 188-212	TMWTDV...WNTGGS PHGGGW...QWNKPS AVVGGL...SRPMIH VTTTTK...RVVEQM
6H4	144-152	DWERRYYR
8H4	147-164	RYYREN...QVYYRP
7A12	122-143	GGLGGY...MIHFGN

Table 1.1- Antigenic amino acid sequences for anti-PrP antibodies. Complied from (Farquhar et al., 1989), (Korth et al., 1997) and (Zanusso et al., 1998). Sequences were derived by PEPSCAN analysis.

```
          10         20         30         40         50         60
MANLGYWLLA LFVTMWTDVG LCKKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGGTWGQPH

          70         80         90        100        110        120
GGGWGQPHGG SWGQPHGGSW GQPHGGGWGQ GGGTHNQWNK PSKPKTNLKH VAGAAAAGAV

         130        140        150        160        170        180
VGGLGGYMLG SAMSRPMIHF GNDWEDRYYR ENMYRYPNQV YYRPVDQYSN QNNFVHDCVN

         190        200        210        220        230        240
ITIKQHTVTT TTKGENFTET DVKMMERVVE QMCVTQYQKE SQAYYDGRRS SSTVLFSSPP

         250
VILLISFLIF LIVG-C-Terminal
```

Figure 1.1- PrP^C sequence and antibody recognition sites. The mouse PrP amino acid sequence was obtained from Swissport accession number P04925 (http://ca.expasy.org.sprot/). Antibody recognition sites have been matched according to information found in the following publications; (Farquhar et al., 1989; Korth et al., 1997; Zanusso et al., 1998). The PrP^C sequence is highlighted according to specific antibody binding sites; blue- 1B3, pink-6H4, green- 8H4 and red-7A12.

Appendix 2: Publication List

Mohan J., Brown K.L., Farquhar C.F., Bruce M.E., Mabbott N.A. (2004). Scrapie transmission following exposure through the skin is dependent on follicular dendritic cells in lymphoid tissues. Journal of Dermatological Science.35, 101-111.

Mohan J., Bruce M.E., Mabbott N.A. (2004). Scrapie neuroinvasion following inoculation via the skin is independent of migratory Langerhans cells. Journal of Virology. Accepted for publication.

Mohan J., Bruce M.E., Mabbott N.A. (2004). Follicular dendritic cells dedifferentiation reduces scrapie susceptibility following inoculation via the skin. Immunology. Accepted for publication.

Bibliography

Acarin, L., Vela, J.M., Gonzalez, B. and Castellano, B. (1994) Demonstation of poly-N-acetyl lactosamine residues in ameboid and ramified microglial cells in rat brain by tomato lectin binding. The Journal of Histochemistry and Cytochemistry 42, 1033-1041.

Adachi, O. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1 and IL-18 mediated function. Immunity 9, 143-150.

Adjou, K.T.R., Demaimay, R., Lasmezas, C.I., Seman, M., Deslys, J.-P. and Dormont, D. (1996) Differential effects of a new amphotericin B derivative, MS-8209, on mouse BSE and scrapie: implications for the mechanism of action of polyene antibiotics. Research Virology 147, 213-218.

Aguzzi, A., Glatzel, M., Montrasio, F., Prinz, M. and Heppner, F.L. (2001) Interventional strategies against prion diseases. Nature Reviews Neuroscience 2, 745-749.

Albert, M.L., Frieda, S., Pearce, A., Francisco, L.M., Sauter, B., Roy, P., Silverstein, R.L. and Bhardwaj, N. (1998) Immature dendritic cells phagocytose apoptotic cells via alpha (v) beta 5 and CD36 and cross-present antigens to cytotoxic T-lymphocytes. Journal of Experimental Medicine 188, 1359-1368.

Alper, T., Haig, D.A. and Clarke, M.C. (1966) The experimentally small size of the scrapie agent. Biochemical and Biophysical Research Communications 22, 278-284.

An, H., Yu, Y., Zhang, M., Xu, H., Qi, R., Yan, X., Liu, S., Wang, W., Guo, Z., Guo, J., Qin, Z. and Cao, X. (2002) Involvement of ERK, p38 and NF-κB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. Immunology 106, 38-45.

Anderson, R.M., Donnelly, C.A., Ferguson, N.M., Woolhouse, M.E.J., Watt, C.J., Udy, H.J., Whinney, S.M.A., Dunstan, S.P., Southwood, M.E.J., Wilesmith, J.W., Ryan, J.B.M., Hoinville, L.J., Hillerton, J.E., Austin, A.R. and Wells, G.A.H. (1996) Transmission dynamics and epidemiology of BSE in British cattle. Nature 382, 779-788.

Andreoletti, O., Berthon, P., Marc, D., Sarradin, P., Grosclaude, J., van Keulen, L., Schelcher, F., Elsen, J.M. and Lantier, F. (2000) Early accumulation of PrP^{Sc} in gut associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. Journal of General Virology 81, 3115-3126.

Antonopoulos, C., Cumberbatch, M., Dearman, R.J., Daniel, R.J., Kimber, I. and Groves, R.W. (2001) Functional Caspase-1 is required for Langerhans cell migration and optimal contact sensitization in mice. Journal of Immunology 166, 3672-3677.

Aucouturier, P., Geissmann, F., Damotte, D., Saborio, G.P., Meeker, H.C., Kascsak, R., Kascsak, R., Carp, R.I. and Wisniewski, T. (2001) Infected splenic dendritic cells are sufficient for prion transmission to the CNS in mouse scrapie. Journal of Clinical Investigation 108, 703-708.

Austyn, J.M., Kupiec-Weglinski, J.W., Hankins, D.F. and Morris, P.J. (1988) Migration patterns of dendritic cells in the mouse. Homing to T-cell-

dependent areas of spleen, and binding within marginal zone. Journal of Experimental Medicine 167, 646-651.

Bacot, S.M., Lenz, P., Frazier-Jenssen, M.R. and Feldman, G.M. (2003) Activation by prion peptide $PrP_{106-126}$ induces a NF-κB-driven proinflammatory response in human monocyte-derived dendritic cells. Journal of Leukocyte Biology 74, 118-125.

Ban, E., Dupre, L., Hermann, E., Rohn, W., Vendeville, C., Quatannens, B., Ricciardi-Castagnoli, P., Capron, A. and Riveau, G. (2000) CpG motifs induce Langerhans cell migration *in vivo*. International Immunology 12, 737-745.

Banchereau, D., Bazan, F. and Blanchard, D. (1994) The CD40 antigen and its ligand. Annual Review of Immunology 12, 881-922.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.-J., Pulendran, B. and Palucka, K. (2000) Immunobiology of dendritic cells. Annual Review of Immunology 18, 767-811.

Banchereau, J., Schuler-Thurner-Thurner, B., Palucka, A.K. and Schuler, G. (2001) Dendritic cells as vectors for therapy. Cell 106, 271-274.

Banchereau, J. and Steinman, R.M. (1998) Dendritic cells and the control of immunity. Nature 392, 245-252.

Barron, R., Thomas, V., Jamieson, E., Melton, D.W., Ironside, J., Will, R. and Manson, J.C. (2001) Changing a single amino acid in the N-terminus of murine PrP alters TSE incubation time across three species barriers. EMBO Journal 20, 5070-5078.

Batista, F.D., Iber, D. and Neuberger, M.S. (2001) B-cells acquire antigen from target cells after synpase formation. Nature 411, 489.

Beekes, M. and McBride, P.A. (2000) Early accumulation of pathological PrP in the enteric nervous system and gut associated lymphoid tissue of hamsters orally infected with scrapie. Neuroscience Letters 278, 181-184.

Beekes, M.P. and McBride, P.A. (1998) Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie. Journal of General Virology 79, 601-607.

Beekes, M.P., McBride, P.A. and Baldauf, E. (1998) Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie. Journal of General Virology 79, 601-607.

Bendheim, P., Barry, R.A., DeArmond, S.J., Stites, D.P. and Pruisner, S.B. (1984) Antibodies to a scrapie prion protein. Nature 310, 418-421.

Beranger, F., Mange, A., Goud, B. and Lehmann, S. (2002) Stimulation of PrP^{C} retrograde transport toward the endoplasmic reticulum increases accumulation of PrP^{Sc} in prion-infected cells. The Journal of Biological Chemistry. 277(41), 38972-38977.

Bergstresser, P.R., Toews, G.B. and Streilein, J.W. (1980) Natural and preturbed distribution of Langerhans cells. Responses to UV light, heterotrophic skin grafting and DNFB sensitization. Journal of Investigative Dermatology 124, 445-453.

Beringue, V., Adjou, K.T., Lamoury, F., Maignien, T., Deslys, J.-P., Race, R. and Dormont, D. (2000a) Opposite effects of dextran sulfate 500, the polyene antibiotic MS-8209, and congo red on accumulation of the protease-resistant

isoform of PrP in the spleens of mice inoculated intraperitoneally with the scrapie agent. Journal of General Virology 74, 5432-5440.
Beringue, V., Demoy, M., Lasmezas, C.I., Gouritin, B., Weingarten, C., Deslys, J.P., Andreux, J.P., Couvreur, P. and Dormont, D. (2000b) Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis. Journal of Pathology 190, 495-502.
Berman, A.E., Kozlova, N.I. and Morozevich, G.E. (2003) Integrins: structure and signaling. Biochemistry 68, 1284-1299.
Berry, M., Bannisters, L.H. and Standring, S.M. (1995) Nervous System. In: P.L. Williams, L.H. Bannister, M.M. Berry, F. Collins, M. Dyson, J.E. Dussek and M.W.J. Ferguson (Eds), Grays Anatomy. Churchill Livingston.
Betmouni, S., Perry, V.H. and Gordon, J.L. (1996) Evidence for an early inflammatory response in the central nervous system of mice with scrapie. Neuroscience 74, 1-5.
Bilsland, C.A., Diamond, M.S. and Springer, T.A. (1994) The leukocyte integrin p150,95 (CD11c/CD18) as a receptor for iC3b; activation by a heterologous beta subunit and localisation of a ligand recognition site to the 1 domain. Journal of Immunology 152, 4582-4589.
Birbeck, M.S., Breathnach, A.S. and Everall, J.D. (1961) An electron microscope study of basal melanocytes and high level clear cells (Langerhans cells) in vitiligo. Journal of Investigative Dermatology 37, 51-64.
Birmingham, K. (2000) Were some CJD victims infected by vaccines? Nature 408, 3-5.
Bolton, D.C., Mc Kinley, M.P. and Prusiner, S.B. (1982) Identification of a protein that purifies with scrapie prion. Science 218, 1309-1311.
Bosma, G.C., Custer, R.P. and Bosma, M.J. (1983) A severe combined immunodeficiency mutation in the mouse. Nature 301, 1339-1347.
Bosque, P.J. and Prusiner, S.B. (2000) Cultured cell sublines highly susceptible to prion infection. Journal of Virology 74, 4377-4386.
Botto, M. (1998) Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nature Genetics 19, 56-59.
Bounhar, Y., Zhang, Y., Goodyer, C. and LeBlanc, A. (2001) Prion protein protects human neurons against Bax-mediated apoptosis. Journal of Biological Chemistry 276, 39145-39149.
Brandner, S., Isenmann, S., Raeber, A., Fischer, E.M., Sailer, A., Kobayashi, Y., Marino, A., Weissmann, C. and Aguzzi, A. (1996) Normal prion protein necessary for scrapie-induced neurotoxicity. Nature 379, 339-343.
Brocker, T., Riedinger, M. and Karjalainen, K. (1997) Targetted expression of major histocompatibility complex (MHC) class molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes *in vivo*. Journal of Experimental Medicine 185, 541-550.
Brotherston, J.G., Renwick, C.C., Stamp, J.T. and Zlotnik, I. (1968) Spread of scrapie by contact to goats and sheep. Journal of Comparative Pathology 78, 9-17.
Brown, D.R. (2004) Role of the prion protein in copper turnover in astrocytes. Neurobiology of Disease 15, 534-543.
Brown, D.R., Qin, K., Herms, J.W., Madling, A. and Manson, J. (1997a) The cellular prion protein binds copper *in vivo*. Nature 390, 684-687.

Brown, D.R., Schulz-Schaeffer, W.J., Schmidt, B. and Kretzschmar, H.A. (1997b) Prion protein deficent cells show altered response to oxidative stress due to decreased SOD-1 activity. Experimental Neurology 146, 104-112.

Brown, K.L., Stewart, K., Bruce, M.E. and Fraser, H. (1997c) Severely combined immunodeficient (SCID) mice resist infection with bovine spongiform encephalopathy. Journal of General Virology. 78, 2707-2710.

Brown, K.L., Stewart, K., Ritchie, D.L., Mabbott, N.A., Williams, A., Fraser, H., Morrison, W.I. and Bruce, M.E. (1999) Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells. Nature Medicine 5, 1308-1312.

Brown, P. (1984) Biologic and chemotherapeutic forays into the feild of unconventional viruses. In: C. De Clercq and R.T. Walker (Eds), Targets for the Design of Antiviral Agents, pp. 131-157. Plenum Press, New York.

Brown, P. and Gajdusek, D.C. (1991) Survival of the scrapie virus after 3 year interment. Lancet 337, 269-270.

Brown, P., Preece, M.A. and Will, R.G. (1992) "Friendly fire" in medicine: hormones, homografts, and Creutzfeldt-Jakob disease. Lancet 340, 24-27.

Brown, P., Rowher, R.G. and Gajdusek, D.C. (1986) New data on the inactivation of scrapie virus or Creutzfeldt-Jakob disease virus in brain tissue. Journal of Infectious Diseases 153, 1145-1148.

Bruce, M.E. (1993) Scrapie strain variation and mutation. In: I.V. Allen (Ed), Spongiform Encephalopathies, pp. 823-826. Vol. 49. Churchill Livingstone.

Bruce, M.E. and Fraser, H. (1991) Scrapie strain variation and its implications. Current Topics in Biological Research 172, 125-138.

Bruce, M.E., McBride, P.A. and Farquhar, C.F. (1989) Precise targetting of the pathology of the sialoglycoprotein, PrP and vacuolar degeneration in mouse scrapie. Neuroscience Letters 102, 1-6.

Bruce, M.E., McBride, P.A., Jeffrey, M. and Scott, J.R. (1994) PrP pathology and pathogenesis in scrapie-infected mice. Molecular Neurobiology 8, 105-112.

Bruce, M.E., Will, R.G., Ironside, J.W., McConnell, I., Drummond, D., Suttie, A., McCardie, L., Chree, A., Hope, J., Birkett, C., Cousens, S., Fraser, H. and Bostock, C.J. (1997) Transmissions to mice indicate that new variant CJD is caused by the BSE agent. Nature 389, 498-501.

Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A. and Autenried, P. (1993) Mice devoid of PrP are resistant to scrapie. Cell 73, 1339-1347.

Bueler, H., Fischer, M., Lang, Y., Bluethmann, H. and Lipp, H.P. (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature 356, 577-582.

Burke, K.E. and Gigli, I. (1980) Receptors for complement on Langerhans cells. Journal of Investigative Dermatology 75, 46-51.

Burwinkel, M., Schwarz, A., Riemer, C., Schultz, J., Van Landcghem, F. and Baier, M. (2004) Rapid disease development in scrapie-infected mice deficient for CD40 ligand. EMBO Reports 5, 527-531.

Campbell, K.A., Ovendale, P.J., Kennedy, M.K., Fanslow, W.C., Reed, S.G. and Maliszewski, C.R. (1996) CD40 ligand is required for protective cell-mediated immunity to Leishmania major. Immunity 4, 283-289.

Cano, G., Sved, A.F., Rinaman, L., Rabin, B.S. and Card, J.P. (2001) Characterization of the central nervous system innervation of the rat spleen

using viral transneuronal tracing. The Journal of Comparative Neurology 439, 1-18.
Carp, R.I. and Callahan, S.M. (1981) *In vitro* interaction of scrapie agent and mouse peritoneal macrophages. Intervirology 16, 8-13.
Carp, R.I. and Callahan, S.M. (1982) Effect of mouse peritoneal macrophages on scrapie infectivity during extended *in vitro* incubation. Intervirology 17, 201-207.
Cashman, N.R., Loertscher, R., Nalbantoglu, J., Shaw, I., Kascsak, R.J., Bolton, S.J. and Bendheim, P.E. (1990) Cellular isoform of the scrapie agent protein participates in lymphocyte activation. Cell 61, 185-192.
Caughey, B., Ernst, D. and Race, R. (1993) Congo red inhibition of scrapie agent replication. Journal of Virology 67, 6270-6272.
Caughey, B. and Race, R. (1992) Potent inhibition of scrapie-associated PrP accumulation by Congo red. Journal of Neurochemistry 59, 768-771.
Caughey, B., Race, R.E., Vogel, M., Buchmeier, M.J. and Chesebro, B. (1988) *In vitro* expression in eukaryotic cells of a prion protein gene extracted from scrapie-infected mouse brain. Proceedings in the National Academy of Science USA 85, 4657-4661.
Caughey, B. and Raymond, G.J. (1993) Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. Journal of Virology 67, 643-650.
Caughey, B., Raymond, G.J., Ernst, D. and Race, R.E. (1991) N-terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of the conversion of PrP to the protease-resistant state. Journal of Virology 65, 6597-6603.
Caughey, B., Raymond, L.D., Raymond, G.J., Maxson, L., Silveira, J. and Baron, G.S. (2003) Inhibiton of protease-resistant prion protein accumulation *in vitro* by curcumin. Journal of Virology 77, 5499-5502.
Caughey, W.S., Raymond, L.D., Horiuchi, M. and Caughey, B. (1998) Inhibition of protease resistant prion protein formation by porphyrins and phthalocyanines. Proceedings of the National Academy of Sciences of the United States of America 95, 12117-12122.
Cella, M. (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T-cell stimulatory capacity: T- help via APC activation. Journal of Experimental Medicine 184, 747-752.
Champion, R.H., Burton, J.L. and Ebling, F.J.G. (1991) Textbook of Dermatology, 373-383 pp. 5th ed, 4. Blackwells Oxford, Oxford.
Chandler, R.L. (1959) Attempts to demonstrate antibodies in scrapie disease. Veterinary Record 71, 58.
Chandler, R.L. (1961) Encephalopathy in mice produced by inoculation with scrapie brain. Lancet 1, 1378-1379.
Chandler, R.L. (1963) Experimental scrapie in mouse. Research in Veterinary Science 4, 276-285.
Chaplin, D.D. and Fu, Y.X. (1998) Cytokine regulation of secondary lymphoid organ development. Current Opinion in Immunology 10, 289-297.
Cho, H.J. (1976) Is the scrapie agent a virus? Nature 262, 411-412.
Cho, H.J. and Greig, A.S. (1975) Isolation of 14-nm virus-like particles from mouse brain infected with scrapie agent. Nature 257, 685-686.

Clarke, A.R. (1968) The antibody response of scrapie-affected mice to immunisation with sheep red blood cells. Research in Veterinary Science 9, 595-597.
Clarke, A.R. and Haig, D.A. (1966) Attempts to demonstrate neutralising antibodies in the sera of scrapie-affected animals. Veterinary Record 78, 647-648.
Clarke, M.C. and Haig, D.A. (1971) Multiplication of scrapie agent in mouse spleen. Research in Veterinary Science 12, 195-197.
Clarke, M.C. and Kimberlin, R.H. (1984) Pathogensis of mouse scrapie:Distribution of agent in the pulp and stroma of infected spleens. Veterinary Microbiology 9, 215-225.
Coe, J.E., Race, R. and Ross, M.J. (2000) Serological evidence for an inflammatory response in murine scrapie. Journal of Infectious Diseases 183, 185-191.
Collee, J.B. and Bradley, R. (1997a) BSE: A decade on. 1. Lancet 349, 636-641.
Collee, J.B. and Bradley, R. (1997b) BSE: A decade on. 2. Lancet 349, 715-721.
Collinge, J., Sidle, K.C.L., Meads, J., Ironside, J. and Hill, A.F. (1996) Molecular analysis of prion strain variation and the aetiology of new variant CJD. Nature 383, 685-690.
Collins, S.J., Lewis, V., Brazier, A.F., Hill, A.F., Fletcher, A. and Masters, C.L. (2002) Quinacrine does not prolong survival time in murine Creutzfeldt-Jakob disease model. Annual Neurology 52, 503-506.
Collis, S.C. and Kimberlin, R.H. (1983) Further studies on changes in immunoglobulin G in the sera and CSF of Herdwick sheep with natural and experimental scrapie. Journal of Comparative Pathology 93, 331-338.
Collis, S.C., Kimberlin, R.H. and Millison, G.C. (1979) Immunoglobulin G concentrations in sera of Herdwick sheep with natural scrapie. Journal of Comparative Pathology 89, 389-396.
Coulon, P., Derbin, C., Kucera, P., Lafay, F., Prehaud, C. and Flamand, A. (1989) Invasion of the perpheral nervous systems of adult mice by the CVS strain of rabies virus and its avirulent derivative AvO1. Journal of Virology 63, 3550-3554.
Cuille, J. and Chelle, P.L. (1936) La maladie dite tremblante du mouton est-elle inocuable? C.R. Acadamy Science 203, 1552-1554.
Cumberbatch, M., Dearman, R.J., Antonopoulos, C., Groves, R.W. and Kimber, I. (2001) Interleukin (IL) 18 induces Langerhans cell migration by tumour necrosis factor alpha and IL-1 beta dependent mechanism. Immunolgy 102, 323-330.
Cumberbatch, M., Dearman, R.J. and Kimber, I. (1997a) Interleukin 1 beta and the stimulation of Langerhans cell migration; comparisons with tumour necrosis factor alpha. Archives in Dermatological Research 289, 277-284.
Cumberbatch, M., Dearman, R.J. and Kimber, I. (1997b) Langerhans cells require signals from both tumour necrosis factor alpha and interleukin 1 beta for migration. Immunology 92, 388-395.
Cumberbatch, M., Dearman, R.J. and Kimber, I. (1999) Langerhans cell migration in mice requires intact type I interleukin 1 receptor (IL-1R1) function. Archives in Dermatological Research 291, 357-361.
Cumberbatch, M. and Kimber, I. (1992) Dermal tumour necrosis factor alpha induces dendritic cells to draining lymph nodes and possibly provides one stimulus for Langerhans cells migration. Immunology 75, 257-263.

Cumberbatch, M., Peters, S.W., Gould, S.J. and Kimber, I. (1992) Intracellular adhesion molecule-1 (ICAM-1) expression by lymph node dendritc cells: comparison with epidermal Langerhans cells. Immunology Letters 32, 105-110.

Cunningham, A.A., Kirkwood, J.K., Dawson, M., Spencer, Y.I., Green, R.B. and Wells, G.A.H. (2004) Distribution of bovine spongiform encephalopathy in greater kudu (Tragelaphus strepsiceros). Emerging Infectious Diseases 10, 1044-1049.

De Smedt, T., Pajak, B., Muraille, L., Lespangnard, E., Heinen, P., De Baetselier, J., Urbain, J., Leo, O. and Moser, M. (1996) Regulation of dendritic cell numbers and maturation by lipopolysaccharide *in vivo*. Journal of Experimental Medicine 184, 1413-1424.

DeArmond, S.J., McKinley, P.M., Barry, R.A., Beckstead, J.H. and Pruisner, S.B. (1985) Identification of prion amyloid filaments in scrapie-infected brains. Cell 41, 221-235.

Deidrich, J., Bendheim, P., Kim, Y.S., Carp, R.I. and Haase, A.T. (1991) Scrapie associated prion protein accumulates in astrocytes during scrapie infection. Proceedings of the National Academy of Sciences of the United States of America 88, 375-379.

Department of Health. (2002) The withdrawal of an oral polio vaccine: analysis of events and implications; a report by the Chief Medical Officer. Department of Health, L. 2002.

Detwiler, L.A., Rubenstein, R. and Williams, E.S. (2000) Transmissible Spongiform Encephalopathies. In: C. Brown and C. Bolin (Eds), Emerging Diseases of Animals, pp. 131-153. ASM Press.

Dickinson, A.G. and Fraser, H. (1979) An assesment of the genetics of scrapie in sheep and mice. In: S.B. Prusiner and W.J. Hadlow (Eds), Slow transmissible diseases of the nervous system., pp. 367-385. Vol. 1. New York Academic Press.

Dickinson, A.G., Fraser, H., McConnell, I. and Outram, G.W. (1978) Mitogenic stimulation of the host enhances susceptibility to scrapie. Nature 272, 54-55.

Dickinson, A.G. and MacKay, J.M.K. (1961) Genetical control of the incubation period in mice of the neurological disease scrapie. Heredity 19, 279-288.

Dickinson, A.G., Meikle, V.M. and Fraser, H. (1969) Genetic control of the concentration of ME7 scrapie agent in the brain of mice. Journal of Comparative Pathology 79, 15-22.

Dickinson, A.G. and Meikle, V.M.H. (1971) Host-genotype and agent effects in scrapie incubation : Change in allelic interaction with different strains of agent. Molecular General Genetics 112, 73-79.

Dickinson, A.G., Meikle, V.M.H. and Fraser, H. (1968) Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. Journal of Comparative Pathology 78, 293-299.

Dickinson, A.G. and Outram, G.W. (1988) Genetic aspects of unconventional virus infections: the basis of the virino hypothesis. Ciba Foundation Symposium 135, 63-83.

Dissanayake, S., Amith, R.S. and Shahin, A. (2004) Taenia crassiceps carbohydrates stimulate IL-6 expression in naive murine macrophages via Toll-like receptors (TLRs). Molecular Immunology 41, 391-398.

Dodelet, V.C. and Cashmann, N.R. (1998) Prion protein expression in human leukocyte differentiation. Blood 91, 1556-1561.

Doh-ura, K., Ishikawa, K., Murakami-Kubo, I., Sasaki, K., Shirou, M., Race, R. and Iwaki, T. (2004) Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. Journal of General Virology 78, 4999-5006.

Duffy, P., Wolf, J., Collins, G., DeVoe, A.G., Streeten, B. and Cowen, D. (1974) Possible person to person transmission of Creutzfeldt-Jacob diseases. New England Journal of Medicine 290, 692.

Dyer, C. (2003) Second vCJD patient to receive experimental treatment. British Medical Journal 327, 886.

Eklund, C.M., Kennedy, R.C. and Hadlow, W.J. (1967) Pathogenesis of scrapie virus infection in the mouse. Journal of Infectious Diseases 117, 15-22.

Elbe, A., Stingl, G., Steiner, G., Tschachler, E., Binder, A. and Wolff, K. (1989) Maturation steps of bone-marrow derived dendritic murine epidermal cells-phenotype and functional studies on Langerhans cells and thy-1 dendritic epidermal-cells in the perinatal period. Journal of Immunology 143, 2431-2438.

Endres, R., Alimzhanov, M.B., Plitz, T., Futterer, A., Kosco-Vilbois, M.H., Nedospasov, S.A., Rajewsky, K. and Pfeffer, K. (1999) Mature follicular dendritic cell networks depend on expression of lymphotoxin and tumour necrosis factor by B-cells. Journal of Experimental Medicine 189, 159-167.

Ermonval, M., Mouillet-Richard, S., Codogno, P., Kellermann, O. and Botti, J. (2003) Evolving views in prion glycosylation: functional and pathological implications. Biochimie 85, 33-45.

Esposito-Farese, M.-E., Sautes, C., de la Salle, H., Latour, S., Bieber, T., de la Salle, C., Olhlmann, P., Fridman, W.H., Cazenave, J.P., Teillaud, J.L., Daeron, M., Bonnerot, C. and Hanau, D. (1995) Membrane and soluble Fc gamma RII/III modulate the antigen presenting capacity of murine dendritic epidermal Langerhans cells for IgG-complexed antigens. Journal of Immunology 154, 1725-1736.

Farquhar, C., Dickinson, A. and Bruce, M.E. (1999) Prophylactic potential of pentosan polysulphate in transmissible spongiform encephalopathies. Lancet 353, 117.

Farquhar, C.F. and Dickinson, A.G. (1986) Prolongation of scrapie incubation Period by an injection of dextran sulphate 500 within the month before or after injection. Journal of General Virology 67, 463-473.

Farquhar, C.F., Dornan, J., Moore, R.C., Somerville, R.A., Tunstall, A.M. and Hope, J. (1996) Protease-resistant PrP deposition in brain and non-central nervous system tissues of a murine model of bovine spongiform encephalopathy. Journal of General Virology 77, 1941-1946.

Farquhar, C.F., Dornan, J., Somerville, R.A., Tunstall, A.M. and Hope, J. (1994) Effect of *Sinc* genotype agent isolate and route of infection on the accumulation of protease-resistant PrP in non-central nervous system tissue during the development of murine scrapie. Journal of General Virology 75, 495-504.

Farquhar, C.F., Somerville, R.A. and Bruce, M.E. (1998) Straining the prion hypothesis. Nature 391, 345-346.

Farquhar, C.F., Somerville, R.A. and Ritchie, L.A. (1989) Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. Journal of Virology Methods 24, 215-222.

Felten, S.Y. and Felten, D.L. (1991) Innervation of lymphoid tissues. Immunological Reviews 100, 225-260.

Fevrier, B., Vilette, D., Archer, F., Loew, D., Faigle, W., Vidal, M., Laude, H. and Raposo, G. (2004) Cells release prions in association with exosomes. Proceedings of the National Academy of Sciences of the United States of America 101, 9683-9688.

Figdor, C.G., van Kooyk, Y. and Adema, G.J. (2002) C-type lectin receptors on dendritic cells and Langerhans cells. Nature Reviews Immunology 2, 77-84.

Flechsig, E., Hegyi, I., Enari, M., Schwarz, P., Collinge, J. and Weissmann, C. (2001) Transmission of scrapie by steel-surface-bound prions. Molecular Medicine 7, 679-684.

Force, W.R., Walter, B.N., Hession, C., Tizard, R., Kozak, C.A., Browning, J.L. and Ware, C.F. (1995) Mouse lymphotoxin-beta receptor. Journal of Immunology 155, 5280-5288.

Foster, J.D., Hunter, N., Williams, A., Mylne, M.J.A., McKelvey, W.A.C., Hope, J., Fraser, H. and Bostock, C. (1996) Observations on the transmission of scrapie in experiments using embryo transfer. Veterinary Record 138, 559-562.

Foster, J.D., Parnham, D.W., Hunter, N. and Bruce, M. (2001) Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. Journal of General Virology 82, 2319-2326.

Fraser, H. (1979) Neuropathology of scrapie: the precision of the lesion and their diversity. In: S.B. Prusiner and W.J. Hadlow (Eds), Slow Transmissible Diseases of the Nervous System, pp. 387-405. Vol. 1. Academic Press, New York.

Fraser, H., Brown, K.L., Stewart, K., McConnell, I., McBride, P. and Williams, A. (1996) Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone marrow. Journal of General Virology 77, 1935-1940.

Fraser, H., Bruce, M.E., Davies, D., Farquhar, C.F. and McBride, P.A. (1992) The lymphoreticular system in the pathogenesis of scrapie. In: S.B. Prusiner, J. Collinge, J. Powell and B. Anderton (Eds), Prion diseases of humans and animals., pp. 308-317. Chichester: Ellis Horwood Ltd.

Fraser, H. and Dickinson, A.G. (1968) Identification of a gene which controls incubation period of some strains of scrapie agent. Journal of Comparative Pathology 78, 301.

Fraser, H. and Dickinson, A.G. (1970) Pathogenesis of scrapie in the mouse: the role of the spleen. Nature 226, 462-463.

Fraser, H. and Dickinson, A.G. (1973) Agent-strain differences in the distribution and intensity of grey matter vacuolation. Journal of Comparative Pathology 83, 29-40.

Fraser, H. and Dickinson, A.G. (1978) Studies of the lymphoreticular system in the pathogenesis of scrapie: The role of the spleen and thymus. Journal of Comparative Pathology 88, 563-573.

Fraser, H. and Farquhar, C.F. (1987) Ionising radiation has no influence on scrapie incubation period in mice. Veterinary Microbiology 13, 211-223.

Fraser, J.R. (1996) Infectivity in extraneural tissues following introcular scrapie infection. Journal of General Virology. 77, 2663-2668.

Fraser, J.R. (2002) What is the basis of transmissible spongiform encephalopathy induced neurodegeneration and can it be repaired? Neuropathology and Applied Neurobiology 28, 1-11.

Fulop, G.M. and Phillips, R.A. (1986) Full reconstitution of the immune deficiency in *scid* mice with normal stem cells requires low-dose irradiation of the recipients. Journal of Immunology 136, 4438-4443.

Fulop, G.M. and Phillips, R.A. (1990) The scid mutation in mice causes a general defect in DNA repair. Nature 347, 479-482.

Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H. and Pfeffer, K. (1998) The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity 9, 59-70.

Gabizon, R., McKinley, M.P., Groth, D. and Prusiner, S.B. (1988) Immunoaffinity purification and neutralization of scrapie prion infectivity. Proceedings of the National Academy of Sciences of the United States of America 85, 6617-6621.

Gajdusek, D.C. (1985) Subacute spongiform virus encephalopathies caused by unconventional viruses. In: K. Marmorosch and J. McKelvey (Eds), Subviral pathogens of plants and animals: Viroids and prions, pp. 483-544. Vol. 1. New York Academic Press.

Garcia Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W. and Thornberry, N.A. (1998) Inhibition of human caspases by peptide-based and macromoleculer inhibitors. Journal of Biological Chemistry 273, 32608-32613.

Gardiner, A.C. and Marucci, A.A. (1969) Immunological responsiveness of scrapie-affected mice. Journal of Comparative Pathology 79, 233-235.

Garfin, D.E., Stites, D.P., Perlman, J.D., Cochran, S.P. and Prusiner, S.B. (1978) Mitogen stimulation of splenocytes from mice infected with scrapie agent. Journal of Infectious Diseases 138, 396-400.

Garret, W.S. and Mellman, I. (1999) Studies of endocytosis. In: M.T. Lotze and A.W. Thomas (Eds), Dendritic Cells, pp. 693-708. Academic Press.

Gerlini, G., Hefti, H.P., Kleinhans, M., Nickoloff, B.J., Burg, G. and Nestle, F.O. (2001) CD1d is expressed on dermal dendritic cells and monocyte derived dendritic cells. Journal of Investigative Dermatology 117, 576-582.

Gilch, S., Winklhofer, K.F. and Groschup, M.H. (2001) Intracellular re-routing of prion protein prevents propagation of PrP^{Sc} and delays onset of prion diseases. EMBO Journal 20, 3957-3966.

Gilch, S., Wopfner, F., Renner-Muller, I., Kremmer, E., Bauer, C., Wolf, E., Brem, G., Groschup, M.H. and Schatzl, H.M. (2003) Polyclonal anti-PrP auto-antiboies induced with dimeric PrP interfere efficiently with PrP^{Sc} propagation in prion-infected cells. Journal of Biological Chemistry 278, 18524-18531.

Glatzel, M., Abela, E., Maissen, M. and Aguzzi, A. (2003) Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease. New England Journal of Medicine 349.

Glatzel, M. and Aguzzi, A. (2000) PrP^C expression in the peripheral nervous system is a determinant of prion neuroinvasion. Journal of General Virology 81, 2813-2821.

Glatzel, M., Heppner, F.L., Albers, K.M. and Aguzzi, A. (2001) Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion. Neuron 31, 25-34.

Goldfarb, L.G., Petersen, R.B., tabaton, M., brown, P., LeBlanc, A., Montagna, P., Cortelli, P., Julien, J., Vital, C. and Pendelbury, W.W. (1992) Fatal familial insomnia and familial Creutzfeldt-Jakob disease: phenotype determined by DNA polymorphism. Science 258, 806-808.

Goldmann, W., Hunter, N., Benson, G., Foster, J.D. and Hope, J. (1991) Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the *Sip* gene. Journal of General Virology 72, 2411-2417.

Goldmann, W., Hunter, N., Smith, N., Foster, J.D. and Hope, J. (1994) PrP genotype and agent effects in scrapie- change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. Journal of General Virology 75, 989-995.

Gommerman, J.L., Giza, K., Perper, S., Sizing, I., Ngam-ek, A., Nickerson-Nutter, C. and Browning, J.L. (2003) A role for surface lymphotoxin in experimental autoimmune encephalomyelitis independent of LIGHT. The Journal of Clinical Investigations. 112, 755-767.

Gordon, W.S. (1946) Advances in veterinary research. Veterinary Research 58, 516-520.

Greig, J.R. (1940) Observations on the transmission of the disease by mediate contact. Veterinary Journal 96, 203-206.

Griffith, J.S. (1967) Self-replication and scrapie. Nature 215, 1043-1044.

Grouard, G., Durand, I., Filgueira, L., Banchereau, J. and Liu, Y.J. (1996) Dendritic cells capable of stimulating T-cells in germinal centers. Nature 384, 364.

Hadlow, W.J. (1959) Scrapie and kuru. Lancet 2, 289-290.

Hadlow, W.J., Eklund, C.M., Kennedy, R.C., Jackson, T.A., Whitford, H.W. and Boyle, C.C. (1974) Course of experimental scrapie infection in goats. Journal of Infectious Diseases 129, 559-567.

Hadlow, W.J., Race, R. and Kennedy, R.C. (1987) Temporal distribution of transmissible mink encephalopathy virus in mink inoculated subcutaneously. Journal of Virology 61, 3235-3240.

Haralambiev, H., Ivanov, I., Vesselinova, A. and Mermerski, K. (1973) An attempt to induce scrapie in local sheep in Bulgaria. Veterinary Medicine 20, 701-709.

Hartsough, G.R. and Burger, D. (1965) Encephalopathy of mink. I. Epizootiologic and clinical observations. Journal of Infectious Diseases. 115, 387-392.

Hay, E.D. (1981) Extracellular matrix. Journal of Cell Biology 91, 205-223.

Hayashi, S., Johnston, S.A. and Takashima, A. (2000) Induction of Th2-directed immune responses by IL-4 transduced dendritic cells in mice. Vaccine 18, 3097-3105.

Head, M., Farquhar, C.F., Mabbott, N.A. and Fraser, J.R. (2001) The transmissible spongiform encephalopathies: pathogenic mechanisms and strategies for therapeutic intervention. Expert Opinion: Therapeutic Targets 2001, 569-585.

Heggebo, R., Press, C.M., Gunnes, G., Gonzalez, L. and Jeffrey, M. (2002) Distribution and accumulation of PrP in gut-associated and peripheral lymphoid tissue of scrapie-affected Suffolk sheep. Journal of General Virology 83, 479-489.

Heggebo, R., Press, C.M., Gunnes, G., Lie, K.I., Tranulis, M.A., Ulvund, M., Groschup, M.H. and Landsverk, T. (2000) Distribution of prion protein in the ileal Peyer's patch of scrapie-free lambs and lambs naturally and experimentally exposed to the scrapie agent. Journal of General Virology 81, 2327-2337.

Heikenwalder, M., Polymenidou, M., Junt, T., Sigurdson, C.J., Wagner, H., Akira, S., Zinkernagel, R. and Aguzzi, A. (2004) Lymphoid follicle destruction and immunosupression after repeated CpG oligodeoxynucleotide administration. Nature Medicine 10, 187-192.

Heller, U., Winklhofer, K.F., Heske, J., Reintjes, A. and Tatzelt, J. (2003) Post-translational import of the prion protein into the endoplasmic reticulum interferes with cell viability. The Journal of Biological Chemistry. 278, 36139-36147.

Hemmi, H., Yoshino, M., Yamazaki, H., Naito, M., Iyoda, T., Omatsu, Y., Shimoyama, S., Letterio, J.J., Nakabayashi, T. and Tagaya, H. (2001) Skin antigens in the steady state are trafficked to regional lymph nodes by transforming growth factor-beta dependent cells. International Immunology 132, 695.

Heppner, F.L., Christ, A.D., Klein, M.A., Prinz, M., Fried, M., Kraehenbuhl, J.-P. and Aguzzi, A. (2001a) Transepithelial prion transport by M-cells. Nature Medicine 7, 976-977.

Heppner, F.L., Musahl, C., Arrighi, I., Klein, A.K., Rulicke, T., Oesch, B., Zinkernage, R.M., Kalinke, U. and Aguzzi, A. (2001b) Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies. Science 294, 178-182.

Hermann, L.M., Cheevers, W.P., Davis, W.C., Knowles, D.P. and O'Rourke, K.I. (2003) CD-21 Positive follicular dendritic cells: A possible source of PrP^{Sc} in lymph node macrophages of scrapie-infected sheep. American Journal of Pathology 162, 1075-1081.

Hertel, L., Lacaille, V.G., Strobl, H., Mellins, E.D. and Mocarski, E.S. (2003) Susceptibility of immature and mature Langerhans cell-type dendritic cells to infection and immunomodulation by human cytomegalovirus. Journal of Virology 77, 7563-7574.

Hill, A.F., Butterworth, R.J., Joiner, S., Jackson, S., Rossor, M.N., Thomas, D.J., Frosh, A., Tolley, N., Bell, J.E., Spencer, M., King, A., Sarraji, S.A., Ironside, J.W., Lantos, P.L. and Collinge, J. (1999) Investigation of variant Creutzfeldt-Jakob diseases and other human prion diseases with tonsil biopsy samples. Lancet 353, 183-189.

Hill, A.F., Desbruslais, M., Joiner, S., Sidle, K.C.L., Gowland, I., Collinge, J., Doey, L.J. and Lantos, P. (1997) The same prion strain causes vCJD and BSE. Nature 389, 448-450.

Hilton, D.A., Fathers, E., Edwards, P., Ironside, J.W. and Zajicek, J. (1998) Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease. Lancet 352, 703-704.

Hilton, D.A., Ghani, A.C., Conyers, L., Edwards, P., McCardle, L., Ritchie, D., Penney, M., Hegazy, D. and Ironside, J. (2004) Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. Journal of Pathology 203, 733-739.

Houston, F., Foster, J.D., Chong, A. and Bostock, C.J. (2000) Transmission of BSE by blood transfusion in sheep. Lancet 356, 999-1000.

Hsiao, K.K., Baker, H.F., Crow, T.J., Poulter, M., Owen, F., Terwilliger, J.D., Westway, D., Ott, J. and Prusiner, S.B. (1989) Linkage of a prion protein missense to Gerstmann-Straussler syndrome. Nature 338, 342-345.

Huang, F.P., Farquhar, C.F., Mabbott, N.A., Bruce, M.E. and MacPherson, G.G. (2002) Migrating intestinal dendritic cells transport PrP^{Sc} from the gut. Journal of General Virology 83, 267-271.

Huang, F.-P., Platt, N., Wykes, M., Major, J.R., Powell, T.J., Jenkins, C.D. and MacPherson, G.G. (2000) A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T-cell areas of mesenteric lymph nodes. Journal of Experimental Medicine 191, 435-443.

Huang, S. (1993) Immune response in mice that lack the interferon gamma receptor. Science 259, 1742-1745.

Hunter, N. (1998) Scrapie. Molecular Biotechnology 9, 225-234.

Hunter, N. (2003) Scrapie and experimental BSE in sheep. British Medical Bulletin 66, 171-183.

Hunter, N., Dann, J.C., Bennett, A.D., Somerville, R.A., McConnell, I. and Hope, J. (1992) Are Sinc and the PrP gene congruent? Evidence from PrP gene analysis in Sinc congenic mice. Journal of General Virology 73, 2751-2755.

Hunter, N. and Houston, F. (2002) Can prion diseases be transmitted between individuals via blood transfusions: Evidence from sheep experiments. Advances in Transfusion Safety 108, 93-98.

Inaba, K., Metlay, J.P., Crowley, M.T. and Steinman, R.M. (1990) Dendritic cells pulsed with protein antigens *in vitro* can prime antigen-specific MHC-restricted T-cells in situ. Journal of Experimental Medicine 172, 631-640.

Inaba, K., Turley, S., Yamaide, F., Iyoda, T., Mahnke, K., Inaba, M., Pack, M., Subklewe, M., Sauter, B., Sheff, D., Albert, M.L., Bhardwaji, N., Mellman, I. and Steinman, R.M. (1998) Efficient presentaion of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. Journal of Experimental Medicine 188, 2163-2173.

Inaba, K., Witmer-Pack, M., Inaba, M., Hathcock, K.S., Sakuta, H., Azuma, M., Yagita, H., Okumura, K., Linsley, P.S. and Ikehara, S. (1994) The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation *in vitro.* Journal of Experimental Medicine 180, 1849-1849.

Ingrosso, L., Ladogana, A. and Pocchiari, M. (1995) Congo red prolongs the incubation period of scrapie-affected hamsters. Journal of Virology 69, 509.

Ingulli, E., Mondino, A., Khoruts, A. and Jenkins, M.K. (1997) *In vivo* detection of dendrtic cell antigen presentation to CD4 T-cells. Journal of Experimental Medicine 185, 2133-2141.

Ironside, J. (2000) Pathology of variant Creutzfeldt-Jakob disease. Archives of Virology 16, 143-151.

Ishii, S., Haga, S., Yagishita, S. and Tateishi, J. (1984) The presence of complements in amyloid plaques of Creutzfeldt-Jakob disease and Gerstmann-Straussler-Scheinker disease. Applied Pathology 2, 370-379.

Jacobson, M.D. and McCarthy, N. (2002) Apoptosis the molecular biology of programmed cell death, 1-16 pp, edited by B.D. Hames and D.M. Glover. Oxford University Press, Oxford.

Jakob, T. and Udey, M.C. (1997) E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells is regulated by cytokines that mobilize Langerhans cells *in vivo*. Journal of Investigative Dermatology 109, 257.

Jeffrey, M., McGoven, G., Martin, S.M., Goodsir, C.M. and Brown, K.L. (2000a) Cellular and sub-cellular localisation of PrP in the lymphoreticular system of mice and sheep. Archives of Virology 16, 23-38.

Jeffrey, M., McGovern, G., Goodsir, C.M., Brown, K.L. and Bruce, M.E. (2000b) Sites of prion protein accumulation in scrapie-infected mouse spleen revealed by immuno-electron microscopy. Journal of Pathology 190, 323-332.

Jeffrey, M. and Wells, G.A. (1988) Spongiform encephalopathy in a nyala (*Tragelaphus angasi)*. Veterinary Pathology 25, 398-399.

Jiang, W. (1995) The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 375, 151-155.

Jung, S., Unutmaz, D., Wong, P., Sano, G.I., De los Santos, K., Sparwasser, T., Wiu, S., Vuthoori, S., Ko, K., Zavala, F., Pamer, E.G., Littman, D.R. and Lang, R.A. (2002) *In vivo* depletion of CD11c dendritic cells abrogates priming of CD8 T cells by exogenous cell-associated antigens. Immunity 17, 211-220.

Kaeser, P.S., Klein, M.A., P., S. and Aguzzi, A. (2001) Efficeint lymphoreticular prion propagation requires PrP^{C} in stromal and hematopoietic cells. Journal of Virology 79, 601-607.

Kaisho, T. and Akira, S. (2003) Regulation of dendritic cell function through toll-like receptors. Current Molecular Medicine 3, 759-771.

Kamperdijk, E.W.A., Dijkstra, C.D. and Dopp, E.A. (1987) Transport of immune complexes from the subcapsular sinus into lymph node follicles of the rat. Immunobiology 174, 395-405.

Kaneider, N.C., Kaser, A., Dunzendorfer, S., Tilg, H. and Wiedermann, C.J. (2003) Sphingosine kinase-dependent migration of immature dendritic cells in response to neurotoxic prion protein fragment. Journal of Virology 77, 5535-5539.

Kapasi, Z.F., Burton, G.F., Schulz, L.D., Tew, J.G. and Szakal, A.K. (1993) Induction of functional follicular dendritic cell development in severe combined immunodeficient mice. Journal of Immunology 150, 2648-2658.

Kaspasi, Z.F., Qin, D., Kerr, W.G., Kosco-Vilbois, M.H., Shultz, L.D., Tew, J.G. and Szakal, A.K. (1998) Follicular dendritic cell (FDC) precursors in primary lymphoid tissues. Journal of Immunology 160, 1078-1084.

Kavanagh, D.G. and Bhardwaj, N. (2002) A division of labour: DC subsets and HIV receptor diversity. Nature Immunology 3, 891-893.

Kawamura, T., Gulden, F.O., Sugaya, M., McNamara, D.T., Borris, D.L., Lederman, M.M., Orenstein, J.M., Zimmerman, P.A. and Blauvelt, A. (2003) R5 HIV productively infects Langerhans cells and infection levels are regulated by compound CCR5 polymorphisms. Proceedings of the National Academy of Sciences of the United States of America 100, 8401-8406.

Kawamura, T., Shimada, S., Yagita, H. and Okumura, K. (1998) FAS/FAS ligand mediated apoptosis of antigen-bearing Langerhans cells in draining lymph nodes. Journal of Investigative Dermatology 110, 488.

Kellermann, S.A., Hudak, S., Oldman, E.R., Liu, Y.J. and Mc Evoy, L.M. (1999) The CC chemokine receptor-7 ligands 6Ckine and macrophage inflammatory protein-3 beta are potent chemoattractants for *in vitro* and *in vivo* derived dendritic cells. Journal of Immunology 162, 3859-3864.

Kiachopoulos, S., Heske, J., Tatzelt, J. and Winklhofer, K.F. (2004) Misfolding of the prion protein at the plasma membrane induces endocytosis, intracellular retention and degradation. Traffic 5, 426-436.

Kim, B.-H., Lee, H.-G., Choi, J.-K., Kim, J., Choi, E.-K., Carp, R.I. and Kim, Y.-S. (2004) The cellular prion protein (PrP^C) prevents apoptotic neuronal cell death and mitochondrial dysfunction induced by serum deprivation. Molecular Brain Research 124, 40-50.

Kimber, I., Cumberbatch, M., Dearman, R.J. and Knight, S.C. (1999) Langerhans cell migration and cellular interaction. In: M.T. Lotze and A.W. Thomas (Eds), Dendritic Cells., pp. 295-310. Academic Press.

Kimberlin, R.H., Field, H. and Walker, C.A. (1983a) Pathogenesis of mouse scrapie: Evidence for spread of infection from central to peripheral nervous system. Journal of General Virology 64, 713-716.

Kimberlin, R.H., Hall, S.M. and Walker, C.A. (1983b) Pathogenesis of mouse scrapie: Evidence for direct neural spread of infection to the CNS after injection of sciatic nerve. Journal of Neurological Science. 61, 315-325.

Kimberlin, R.H. and Walker, C.A. (1979) Pathogenesis of mouse scrapie: dynamics of agent replication in the spleen, spinal cord and brain after infection by different routes. Journal of Comparative Pathology 89, 551-562.

Kimberlin, R.H. and Walker, C.A. (1980) Pathogenesis of mouse scrapie: Evidence for neural spread of the infection to the CNS. Journal of General Virology 51, 183-187.

Kimberlin, R.H. and Walker, C.A. (1989a) Pathogenesis of scrapie in mice after intragastric infection. Virus Research 12, 213-220.

Kimberlin, R.H. and Walker, C.A. (1989b) The role of the spleen in the neuroinvasion of scrapie in mice. Virus Research 12, 201-212.

Kimberlin, R.H. and Walker, C.A. (1990) Intraperitoneal infection with scrapie is established within minutes of injection and is non-specifically enhanced by a variety of different drugs. Archives of Virology 112, 103-114.

Kimberlin, R.H., Walker, D.G., Millson, G.C., Taylor, D.M., Robertson, P.A., Tomlinson, A.H. and Dickinson, A.G. (1983c) Disinfection studies of two stains of mouse-passaged scrapie agent. Guidlines for Creutzfeldt-Jakob and related agents. Journal of Neuroscience 59, 355-369.

Kingsbury, D.T., Smeltzer, D.A., Clarence, J., Gibbs, J.R. and Gajdusek, D.C. (1981) Evidence for normal cell-mediated immunity in scrapie-infected mice. Infection and Immunity 32, 1176-1180.

Kirkwood, J.K., Wells, G.A.H., Wilesmith, J.W., Cunningham, A.A. and Jackson, S.I. (1990) Spongiform encephalopathy in an arabian oryx (*Oryx leucoryx*) and a greater kudu (*Tragelaphus strepsiceros)*. Veterinary Record 127, 418-420.

Kitamoto, T., Muramoto, T., Mohri, S., Doh-Ura, K. and Tateishi, J. (1991) Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. Journal of Virology 65, 6292-6295.

Kitamura, D., Roes, J., Kuhn, R. and Rajewsky, K. (1991) A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature 350, 423-426.

Klareskog, L., Tjernlund, U., Forsum, U. and Peterson, P.A. (1977) Epidermal Langerhans cells express Ia antigens. Nature 268, 248-250.

Klein, A.K., Frigg, R., Flechsig, E., Raeber, A.J., Kalinke, U., Bluethmanns, H., Bootz, F., Suter, M., Zinkernage, R.M. and Aguzzi, A. (1997) A crucial role for B-cells in neuroinvasive scrapie. Nature 390, 687-690.

Klein, M.A., Frigg, R., Raeber, A.J., Flechsig, E., Hegyi, I., Zinkernagel, R.M., Weissmann, C. and Aguzzi, A. (1998) PrP expression in B-lymphocytes is not required for prion neuroinvasion. Nature Medicine 4, 1429-1433.

Klein, M.A., Kaeser, P.S., Schwarz, P., Weyd, H., Xenarios, I., Zinkernagel, R.M., Carroll, M.C., Verbeek, J.S., Botto, M., Walport, M.J., Molina, H., Kalinke, U., Acha-Orbea, H. and Aguzzi, A. (2001) Complement Facilitates early prion pathogenesis. Nature Medicine 7, 488-492.

Kligman, A.M. (1964) The biology of the stratum corneum. In: W. Montagna and W.C. Lobitz (Eds), The epidermis, pp. 387-433. Academic Press, New York.

Kobayashi, Y. (1997) Langerhans cells produce type IV collagenase (MMP-9) following epicutaneous stimulation with haptens. Immunology 90, 496-501.

Koch, F. (1996) High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD 40 molecules and downregulation by IL-4 and IL-10. Journal of Experimental Medicine 184, 741-747.

Kodali, S., Friedman, I., Ding, W., Seiffert, K., Wagner, J.A. and Granstein, R.D. (2003) Pituitary adenylate cyclase-activating polypeptide inhibits cutaneous immune function. European Journal of Immunology 33, 3070-3079.

Koni, P.A., Sacca, R., Lawton, P., Browning, J.L., Ruddle, N.H. and Flavell, R.A. (1997) Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta deficient mice. Immunity 6, 491-500.

Korth, C., May, B.C.H., Cohen, F.E. and Prusiner, S.B. (2001) Acridine and phenothiazine derivatives as pharmacotherapeutics for prion diseases. Proceedings of the National Academy of Sciences of the United States of America 98, 9836-9841.

Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzchmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S., Glaockshuber, R., Riek, R., Billeter, M., Wuthrich, K. and Oesch, B. (1997) Prion protein (PrP^{sc})- specific epitope defined by a monoclonal antibody. Nature 390, 74-77.

Kosco-Vilbois, M.H., Zentgraf, H., Gerdes, J. and Bonncfoy, J.-Y. (1997) To B or not to B a germinal center? Immunology Today 18, 225-230.

Koster, T., Singh, K., Zimmermann, M. and Gruys, E. (2003) Emerging therapeutic agents for transmissible spongiform encephalopathies: a review. Journal of Veterinary Pharmacology and Therapeutics 26, 315-326.

Kovacs, G.G., Gasque, P., Strobel, T., Lindeck-Pozza, E., Strohschneider, M., Ironside, J.W., Budka, H. and Guentchev, M. (2004) Complement activation in human prion diseases. Neurobiology Diseases 15, 21-28.

Kubosaki, A., Nishimura-Nasu, Y., Nishimura, T., Yusa, S., Sakudo, A., Saeki, K., Matsumoto, Y., Itohara, S. and Onodera, T. (2003) Expression of normal cellular prion protein (PrP^C) on T-lymphocytes and the effect of copper ion: analysis by wild-type and prion protein gene-deficient mice. Biochemical and Biophysical Research Communications 307, 810-813.

Kupper, T.S. and Fuhlbrigge, R.C. (2004) Immune surveillance in the skin:mechanisms and clinical consequences. Nature Reviews Immunology 4, 211-222.

Kurimoto, I., Arana, M. and Streilein, J.W. (1994) Role of dermal cells from normal and ultraviolet B-damaged skin in the induction of contact hypersensitivity. Journal of Immunology 152, 3317-3323.

Kuwahara, C., Takeuchi, A.M., Nishimura, T., Haraguchi, K., Kubosaki, A., Matsumoto, Y., Saeki, K., Matsumoto, Y., Yokoyama, T., Itohara, S. and Onodera, T. (1999) Prions prevent neuronal cell-line death. Nature 400, 225-226.

Ladogana, A., Casaccia, P. and Ingrosso, L. (1992) Sulphate polyanions prolong the incubation period of scrapie infected hamsters. Journal of General Virology 73, 661-665.

Langerhans, P. (1868) Uber die nerven der menschlichen haut. Virchows Arch (Pathol Arch.) 44, 325-337.

Lappin, M.B., Kimber, I. and Norval, M. (1996) The role of dendritic cells in cutaneous immunity. Archives of Dermatological Research 288, 109-121.

Larregina, A.T., Morelli, A.E., Spencer, L.A., Logar, A.J., Watkins, S.C., Thomson, A.W. and Falo, L.D. (2001) Dermal-resident CD14+ cells differentiate into Langerhans cell. Nature Immunology 2, 1151-1158.

Lasmezas, C.I., Cesbron, J.-Y., Deslys, J.P., Demaimay, R., Adjou, K.T., Riox, R., Lemarie, C., Locht, C. and Dormont, D. (1996) Immune system dependent and independent replication of the scrapie agent. Journal of Virology 70, 1292-1295.

Lasmezas, C.I., Deslys, J.P., Robain, O., Jaegly, A., Beringue, V., Peyrin, J.M., Hauw, J., Rossier, J. and Dormont, D. (1997) Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. Science 275, 402-405.

Lee, H.S., Brown, P., Cervenakova, L., Garruto, R.M., Alpers, M.P., Gajdusek, D.C. and Goldfarb, L.G. (2001) Increased susceptibility to Kuru of carriers of the PRNP 129 methionine/methionine genotype. Journal of Infectious Diseases 183, 192-196.

Lee, I.Y., Westway, D., Smit, A.F., Wang, K., Seto, J., Chen, L., Acharya, C., Ankener, M., Baskin, D., Cooper, C., Yao, H., Pruisner, S.B. and Hood, L.E. (1998) Complete genomic sequence and analysis of the prion protein gene region from three mammalian species. Genome Research 8, 1022-1037.

Legname, G., Baskakov, I.V., Nguyen, H.-O.B., Riesner, D., Cohen, F.E., DeArmond, S.J. and Pruisner, S.B. (2004) Synthetic mammalian prions. Science 305, 673-676.

Lewicki, H., Tishon, A., Homann, D., Mazarguil, H., Laval, F., Asensio, V.C., Campbell, I.L., DeArmond, S.J., Coon, B., Teng, C., Gairin, J.E. and Oldstone, M.B.A. (2003) T-cells infiltrate the brain in murine and human

transmissible spongiform encephalopathies. Journal of Virology 77, 3799-3808.

Lin, Y., Lee, H.S., Berg, A.H., Lisanti, M.P., Shapiro, L. and Scherer, P.E. (2000) The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces sysnthesis of the closely related TLR-2 in adipocytes. Journal of Biological Chemistry 275, 24255-24263.

Llewelyn, C.A., Hewitt, P.E., Knight, R.S.G., Amar, K., Cousens, S., Mackenzie, J. and Will, R.G. (2004) Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet 363, 417-421.

Lloyd, S.E., Thompson, S.R., Beck, J.A., Linehan, J.M., Wadsworth, J.D.F., Brandner, S., Collinge, J. and Fisher, E.M. (2004) Identification and characterization of a novel mouse prion gene allele. Mammalian Genome 15, 383-389.

Lotscher, M., Recher, M., Hunzinker, L. and Klein, M.A. (2003) Immunologically induced complement-dependent up-regulation of the prion protein in the mouse spleen: follicular dendritic cells versus capsule and trabeculae. Journal of Immunology 170, 6040-6047.

Lu, M., Zhang, M., Kitchens, R.L., Fosmire, S., Takashima, A. and Munford, R.S. (2003) Stimulus-dependent deacylation of bacterial lipopolysaccharide by dendritic cells. Journal of Experimental Medicine 197, 1745-1754.

Ludewig, B. (2003) Dendritic cell vaccination and viral infection- animal models. Current Topics in Microbiology and Immunology 276, 199-214.

Luger, T.A. and Schwarz, A. (1990) Evidence for an epidermal cytokine network. Journal of Investigative Dermatology 95, 100-104.

Luhr, K.M., Nordstom, E.K., Low, P., Ljunggren, H.-G., Taraboulos, A.T. and Kristensson, K. (2004) Scrapie protein degradation by cysteine proteases in CD11c+ dendritic cells and GT1-1 neuronal cells. Journal of Virology 78, 4776-4782.

Luhr, K.M., Wallin, R.P.A., Ljunggren, H.-G., Low, P., Taraboulos, A.T. and Kristensson, K. (2002) Processing and degradation of exogenous prion protein by CD11c+ myeloid dendritic cells in vitro. Journal of Virology 76, 12259-12264.

Lupi, O. (2002) Prions in dermatology. Journal of American Academy of Dermatology 46, 790-793.

Lupi, O. (2003) Could ectoparasites act as vectors for prion diseases? International Journal of Dermatology 42, 425-429.

Ma, J. and Lindquist, S. (2001) Wild-type PrP and a mutant associated with prion disease are subject to retrograde transport and proteasome degradation. Proceedings in the National Academy of Science USA 98, 14955-14960.

Ma, J., Wollmann, R. and Lindquist, S. (2002) Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. Science 298, 1781 1785.

Ma, X. (2001) TNF-α and IL-12: a balancing act in macrophage functioning. Microbes and Infection 3, 121-129.

Mabbott, N.A., Brown, K.L., Manson, J.C. and Bruce, M.E. (1997) T-lymphocyte activation and the cellular form of the prion protein. Immunology 92, 161-165.

Mabbott, N.A. and Bruce, M.E. (2001) The immunobiology of TSE diseases. Journal of General Virology 82, 2307-2318.

Mabbott, N.A., Bruce, M.E., Botto, M., Walport, M.J. and Pepys, M.B. (2001) Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie. Nature Medicine 7, 485-487.

Mabbott, N.A., Farquhar, C.F., Brown, K.L. and Bruce, M.E. (1998) Involvement of the immune system in TSE pathogenesis. Immunology Today 19, 201-203.

Mabbott, N.A., Mackay, F., Minns, F. and Bruce, M.E. (2000a) Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. Nature Medicine 6, 719-720.

Mabbott, N.A., McGoven, G., Jeffrey, M. and Bruce, M.E. (2002) Temporary blockade of the tumour necrosis factor receptor signaling pathway impedes the spread of scrapie to the brain. Journal of Virology 76, 1-9.

Mabbott, N.A., Williams, A., Farquhar, C.F., Pasparakis, M., Kollias, G. and Bruce, M.E. (2000b) Tumour necrosis factor alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie. Journal of Virology 74, 3338-3344.

Mabbott, N.A., Young, J., McConnell, I. and Bruce, M.E. (2003) Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility. Journal of Virology 77, 6845-6854.

MacDonald, G.H. and Johnston, R.E. (2000) Role of dendritic cell targeting in Venezuelan Equine Encephalitis virus pathogenesis. Journal of Virology 74, 914-922.

MacGregor, I.R., Dawes, J., Paton, L., Pepper, D.S., Prowse, C.V. and Smith, M.A. (1984) Metabolism of sodium pentosan polysulphate in man-catabolism of iodinated derivaties. Thrombosis and Haemostasis 51, 321-325.

Mackay, F. and Browning, J.L. (1998) Turning off Follicular dendritic cells. Nature 395, 26-27.

Mange, A., Crozet, C., Lehmann, S. and Beranger, F. (2004) Scrapie-like prion protein is translocated to the nuclei of infected cells independently of the proteasome inhibition and interacts with chromatin. Journal of Cell Science 117, 2411-2416.

Manson, J., West, J.D., Thomson, V., McBride, P., Kaufman, M.H. and Hope, S. (1992) The prion protein gene: A role in mouse embryogenesis. Development 115, 117-122.

Manson, J.C., Clarke, A.R., Hooper, M.L., Aitchison, L., McConnell, I. and Hope, J. (1994a) 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. Molecular Neurobiology 8, 121-127.

Manson, J.C., Clarke, A.R., McBride, P.A., McConnell, I. and Hope, J. (1994b) PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology. Neurodegeneration 3, 331-340.

Manson, J.C., Jamieson, E., Baybutt, H., Tuzi, N.L., Barron, R., McConnell, I., Somerville, R.A., Ironside, J., Will, R., Man-Sun, S., Melton, D.W., Hope, J. and Bostock, C. (1999) A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. EMBO Journal 18, 6855-6864.

Marriott, I., Thomas, E.K. and Bost, K.L. (1999) CD40-CD40 ligand interactions augment survival of normal mice, but not CD40 ligand knockout mice,

challenged orally with *Salmonella dublin*. Infection and Immunity 67, 5253-5257.

Marsh, R.F. and Hanson, R.P. (1971) The syrian hamster as a model for the study of slow virus disease caused by unconventional agents. Federal Proceedings. 37, 2076-2078.

Marsh, R.F., Pan, I.C. and Hanson, R.P. (1970) Failure to demonstrate specific antibody in transmissible mink encephalopathy. Infection and Immunity 2, 727-730.

Martins, V.R., Linden, R., Prado, M.A.M., Walz, R., Sakamoto, A.C., Izquierdo, I. and Brentani, R.R. (2002) Cellular prion protein: on the road for functions. FEBS Letters 512, 25-28.

Matsue, H., Matsue, K., Walters, M., Okumura, K., Yagita, H. and Takashima, A. (1999) Induction of antigen-specific immunosuppression by CD59L c-DNA-transfected "killer" dendritic cells. Nature Medicine 5, 930-937.

Matsumoto, M., Lo, D., Carruthers, C.J.L., Min, J., Mariathasan, S., Huang, G., Plas, D.R., Martin, S.M., Geha, R.S., Nahm, M.N. and Chaplin, D.D. (1996a) Affinity maturation without germinal centres in lymphotoxin alpha-deficient mice. Nature. 382, 462-466.

Matsumoto, M., Mariathasan, S., Nahm, M.N., Baranyay, F., Peschon, J.J. and Chaplin, D.D. (1996b) Role of lymphotoxin and the type 1 TNF receptor in the formation of germinal centers. Science 271, 1289-1291.

Mauri, D.N., Ebner, R., Montgomery, R.I., Kochel, K.D., Cheung, T.C., Yu, G.L., Ruben, S., Murphy, M., Eisenberg, R.J., Cohen, G.H., Spear, P.G. and Ware, C.F. (1998) LIGHT, a new member of the TNF superfamily and lymphotoxin alpha are ligands for herpesvirus entry mediator. Immunity 8, 21-30.

Mayor, S. (2003) Small improvemnt seen in teenager with vCJD. British Medical Journal 327, 765.

McBride, P.A. and Beekes, M.P. (1999) Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie. Neuroscience Letters 265, 135-138.

McBride, P.A., Eikelenboom, P., Kraal, G., Fraser, H. and Bruce, M.E. (1992) PrP protein in associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie infected mice. Journal of Pathology 168, 413-418.

McBride, P.A., Schulz-Schaeffer, W., Donaldson, M., Bruce, M.E., Diringer, H., Kretzchmar, H. and Beekes, M.P. (2001) Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibers of the splanchnic and vagus nerves. Journal of Virology 75, 9320-9327.

McGovern, G., Brown, K.L., Bruce, M.E. and Jeffrey, M. (2004) Murine scrapie infection causes an abnormal germinal centre reaction in the spleen. Journal of Comparative Pathology 130, 181-194.

McGowan, J.P. (1922) Scrapie in sheep. Scottish Journal of Agriculture 5, 365-375.

Mertz, P.A., Somerville, R.A., Wisneiwski, H.M. and Iqbal, K. (1981) Abnormal fibrils from scrapie infected brain. Acta Neuropathol 54, 63-74.

Meyer, R.K., Mc Kinley, M.P., Bowman, K.A., Braunfeld, M.B., Barry, R.A. and Prusiner, S.B. (1986) Separation and properties of cellular and scrapie prion proteins. Proceedings of the National Academy of Sciences of the United States of America 83, 2310-2314.

Mignini, F., Streccioni, V. and Amenta, F. (2003) Autonomic innervation of immune organs and neuroimmune modulation. Autonomic and Autacoid Pharmacology 23, 1-25.

Milhavet, O., McMahon, H.E.M., Rachidi, W., Nishida, N., Katamine, S., Mange, A., Arlotto, M., Casanova, D., Riondel, J., Favier, A. and Lehmann, S. (2000) Prion infection impairs the cellular response to oxidative stress. Proceedings of the National Academy of Sciences of the United States of America 97, 13937-13942.

Miller, M.W., Williams, E.S., Hobbs, N.T. and Wolfe, L.L. (2004) Environmental sources of prion transmission in mule deer. Emerging Infectious Diseases 10, 1003-1006.

Millhauser, G.L. (2004) Copper binding in the prion protein. Accounts of Chemical Research 37, 79-85.

Millington, P.F. and Wilkinson, R. (1983) Skin. In: R. Harrison and R.M.H. McMinn (Eds), Biological structure and function. Vol. 9. Cambridge University Press, Cambrigde.

Mitsui, H., Watanabe, T., Saeki, H., Mori, N., Fujita, H., Tada, Y., Asahina, A., nakamura, K. and Tamaki, K. (2004) Differential expression and function of Toll-like receptors in Langerhans cells: comparision with splenic dendritic cells. Journal of Investigative Dermatology 122, 95-102.

Mizumoto, N., Kumamoto, T., Robson, S.C., Sevigny, J., Matsue, H., Enjyoji, K. and Takashima, A. (2002) CD39 is the dominant Langerhans cell-associated ecto-NTPDase; modulatory roles in inflammation and immune responsiveness. Nature Medicine 8, 358-365.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S. and Papaioannou, V.E. (1992) RAG-1 deficient mice have no mature B and T lymphocytes. Cell 68, 869-877.

Mommaas, M., Mulder, A., Vermeer, B.J. and Koning, F. (1994) Functional human epidermal Langerhans cells that lack Birbeck granules. Journal of Investigative Dermatology 103, 807-810.

Montrasio, F., Cozzio, A., Flechsig, E., Rossi, D., Klein, M.A., Rulicke, T., Raeber, A., Vosshenrich, C.A.J., Proft, J., Aguzzi, A. and Weissmann, C. (2001) B lymphocyte-restricted expression of prion protein does not enable prion replication in prion protein knockout mice. Proceedings in the National Academy of Science USA 98, 4034-4037.

Montrasio, F., Frigg, R., Glatzel, M., Klein, M.A., Mackay, F., Aguzzi, A. and Weissmann, C. (2000) Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. Science 288, 1275-1259.

Moodycliffe, A.M., Shreedhar, V., Ullrich, S.E., Walterscheid, J., Bucana, C., Kripke, M.L. and Flores-Romo, L. (2000) CD40-CD40 ligand interactions *in vivo* regulate migration of antigen-bearing dendritic cells from the skin to draining lymph nodes. Journal of Experimental Medicine 191, 2011-2020.

Moore, R.C., Hope, J., McBride, P.A., McConnell, I., Selfridge, J., Melton, D.W. and Manson, J.C. (1998) Mice with gene targetted prion protein alterations show that *Prnp, Sinc* and *Prni* are congruent. Nature Genetics 18, 118-125.

Murakami, -.K., I., Doh-ura, K., Ishikawa, K., Kawatake, S., Sasaki, K., Kira, J.-I., Ohta, S. and Iwaki, T. (2004) Quinoline derivatives are therapeutic

candidates for transmissible spongiform encephalopathies. Journal of Virology 78, 1281-1288.

Nakajima, M., Yamada, T., Kusuhara, T., Furukawa, H., Takahashi, M., Yamauchi, A. and Kataoka, Y. (2004) Results of quinacrine administration to patients with Creutzfeldt-Jakob disease. Dementia and Geriatric Cognitive Disorders 17, 158-163.

Neutra, M.R., Mantis, N.J. and Kraehenbuhl, J.-P. (2001) Collaboration of epithelial cells with organised mucosal lmphoid tissues. Nature Immunology 2, 1004-1009.

Nielsen, C.H., Fischer, E.M. and Leslie, R.G.Q. (2000) The role of complement in the acquired immune response. Immunology 100, 4-12.

Nolte, M.A., Belien, J.A.M., Schadee-Eestermans, I., Jansen, W., Unger, W.W.J., van Rooijen, N., Kraal, G. and Mebius, R.E. (2003) A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. Journal of Experimental Medicine 198, 505-512.

Nossal, G.J., Abbbot, A., Mitchell, J. and Lummus, Z. (1968a) Antigens in immunity; Ultrastructural features of antigen capture in primary and secondary lymphoid follicules. Journal of Experimental Medicine 127, 277-290.

Nossal, G.J., Abbot, A. and Mitchell, J. (1968b) Antigens in immunity. XIV. Electron microscopic radioautographic studies of antigen capture in the lymph node medulla. Journal of Experimental Medicine 127, 263-276.

Oesch, B., Westway, P. and Walchli, M. (1985) A cellular gene encodes scrapie PrP_{27-30} protein. Cell 40, 735-746.

Oldstone, M.B.A., Race, R., Thomas, D., Lewicki, H., Homann, D., Smelt, S., Holz, A., Koni, P., Lo, D., Chesebro, B. and Flavell, R.A. (2002) Lymphotoxin-alpha and lymphotoxin-beta deficient mice differ in susceptibility to scrapie: Evidence against dendritic cell involvement in neuroinvasion. Journal of Virology 76, 4357-4363.

O'Rourke, K.I., Huff, T.P., Leathers, C.W., Robinson, M.M. and Gorham, J.R. (1994) SCID mouse spleen does not support scrapie agent replication. Journal of General Virology 75, 1511-1514.

Outram, G.W., Dickinson, A.G. and Fraser, H. (1974) Reduced susceptibility to scrapie in mice after steroid administration. Nature 249, 855-856.

Paczesny, S., Ueno, H., Fay, J., Banchereau, J. and Palucka, A.K. (2003) Dendritc cells as vectors for immunotherapy of cancer. Seminars in Cancer Biology 13, 439-447.

Palsson, P.A. (1979) Rida (scrapie) in Iceland and its epidemiology. In: S.B. Prusiner and W.J. Hadlow (Eds), Slow Transmissible Diseases of the Nervous System, pp. 357-366. Vol. 1. New York Academic Press.

Pammer, J. and Tschachler, E. (2002) A possible role of keratinocytes of skin and mucous membranes in prion propagation and transmission. Journal of Investigative Dermatology 7, 59-63.

Pammer, J., Weninger, W. and Tschachler, E. (1998) Human keratinocytes express cellular prion-related protein *in vitro* and during inflammatory skin diseases. American Journal of Pathology 153, 1353-1358.

Pan, K.M., Baldwin, M., Nguyen, J., Gassal, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E. and Prusiner, S.B. (1993) Conversion

of alpha helices into beta sheets features in the formation of the scrapie prion proteins. Proceedings in the National Academy of Science USA 90, 10962-10966.

Paramithiotis, E., Pinard, M., Lawton, T., LaBoissiere, S. and Leathers, V.L. (2003) A prion protein epitope selective for the pathologically misfolded conformation. Nature Medicine 9, 893-899.

Parchi, P., Castellani, R., Capellari, S., Petersen, R.B., Chen, S.G., Young, K., Farlow, M., Trojanowski, J.Q., Sima, A., Ghetti, B. and Gambetti, P. (1995) Protease-resistant prion protein in sporadic Creutzfeldt-Jakob disease (CJD): correlation with clinical pathological features and PrP genotype. Journal of Neuropathology and Experimental Neurology 54, 416.

Pasparakis, M., Alexopoulo, L., Episkopou, V. and Kollias, G. (1996) Immune and inflammatory responses in TNF alpha in the formation of primary B-cell follicles, follicular dendritic cell networks, and germinal centers and in the maturation of the humoral response. Journal of Experimental Medicine 184, 1397-1411.

Pattison, I., Millson, G.C. and Smith, K. (1964) An examination of the action of whole blood, blood cells or serum on the goat scrapie agent. Research in Veterinary Science 5, 116-121.

Pepys, M.B. (1976) Role of complement in the induction of immunological responses. Transplant Reviews 32, 93-120.

Peretz, D., Williamson, R.A. and Kaneko, K. (2001) Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. Nature 412, 739-743.

Perrier, V., Solassol, J., Crozet, C., Frobert, Y., Mourton-Gilles, C., Grassi, J. and Lehmann, S. (2004) Anti-PrP antibodies block PrP^{Sc} replication in prion-infected cell cultures by accelerating PrP^{C} degradation. Journal of Neurochemistry 89, 454-463.

Phillips, R.A. and Fulop, G.M. (1989) Pleiotropic effects of the *scid* mutation: effects on lymphoid differentiation and on repair of radiation damage. In: M.J. Bosma, R.A. Phillips and G. Schuler (Eds), The Scid Mouse: Characterisation and Potential Uses, pp. 11-17. Springer-Verlag.

Pincock, S. (2004) Government confirms second case of vCJD transmitted by blood transfusion. British Medical Journal 329, 251.

Pocchiari, M., Schmittingers, S. and Masullo, C. (1987) Amphotericin B delays the incubation perios of scrapie in intracerebrally inoculated hamsters. Journal of General Virology 68, 219-223.

Porter, D.D., Porter, H.G. and Cox, N.A. (1973) Failure to demonstrate a humoral immune response to scrapie infection in mice. Journal of Immunology 111, 1407-1410.

Post, K., Riesner, D., Walldorf, V. and Mehlhorn, R. (1999) Fly larvae and pupae as vectors for scrapie. Lancet 122, 199-204.

Pouniotis, D.S., Proudfoot, O., Minigo, G., Hanley, J.L. and Plebanski, M. (2004) Malaria parasite interactions with the human host. Journal of Postgraduate Medicine 50, 30-34.

Price, A.A., Cumberbatch, M., Kimber, I. and Ager, A. (1997) Alpha 6 integrins are required for Langerhans cell migration from the epidermis. Journal of Experimental Medicine 186, 1725-1735.

Prinz, M., Heikenwalder, M., Schwarz, P., Takeda, K., Akira, S. and Aguzzi, A. (2003a) Prion pathogenesis in the absence of toll-like receptor signalling. EMBO reports 4, 195-199.

Prinz, M., Helkenwalder, M., Junt, T., Schwarz, P., Glatzel, M., Heppner, F.L., Fu, Y.-X., Lipp, M. and Aguzzi, A. (2003b) Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion. Nature 425, 957-962.

Prinz, M., Montrasio, F., Klein, M.A., Schwarz, P., Priller, J., Odermatt, B., Pfeffer, K. and Aguzzi, A. (2002) Lymph nodal prion replication and neuroinvasion of follicular dendritic cells. Proceedings of the National Academy of Sciences of the United States of America 99, 919-924.

Priola, S.A., Caughey, B., Wehrly, K. and Chesebro, B. (1995) A 60-kDa prion protein (PrP) with properties of both the normal and scrapie-associated forms of PrP. The Journal of Biological Chemistry. 270, 3299-33054.

Prusiner, S.B. (1982) Novel proteinaceous infectious particles cause scrapie. Science 216, 136-144.

Prusiner, S.B. (1991) Molecular biology of prion diseases. Science 252, 1515-1522.

Prusiner, S.B. (1998) Prions. Proceedings of the National Academy of Sciences of the United States of America 95, 13363-13383.

Race, R., Priola, S.A., Bessen, R.A., Ernst, D., Dockter, J., Rall, G.F., Mucke, L., Chesebro, B. and Oldstone, M.B.A. (1995) Neuron-specific expression of a hamster prion protein minigene in transgeneic mice induces susceptibility to hamster scrapie agent. Neuron 15, 1183-1191.

Raeber, A., Sailer, A., Hegyi, I., Klein, M.A., Rulicke, T., Fischer, M., Brandner, S., Aguzzi, A. and Weissmann, C. (1999a) Ectopic expression of prion protein (PrP) in T-lymphocytes or hepatocytes of PrP knockout mice is sufficient to sustain prion replication. Proceedings of the National Academy of Sciences of the United States of America 96, 3987-3992.

Raeber, A.J., Klein, M.A., Frigg, R., Flechsig, E., Aguzzi, A. and Weissmann, C. (1999b) PrP-dependent association of prions with splenic but not circulating lymphocytes of scrapie-infected mice. EMBO Journal 18, 2702-2706.

Ramasamy, I., Law, M., Collins, S.J. and Brooke, F. (2003) Variant Creutzfeldt-Jakob disease and the potential for its accidental transmisssion following surgery with contaminated instruments: the risk of transmission in Australia. Folia Neuropathologica 41, 1-10.

Raposo, G., Nijman, H.W., Stoorvogel, W., Liejendekker, R., Harding, C.V. and Geuze, H.J. (1996) B-lymphocytes secrete antigen presenting vesicles. Journal of Experimental Medicine 183, 1161-1172.

Reece, J.C., Handley, A.J., Anstee, E.J., Morrison, W.A., Crowe, S.M. and Cameron, P.U. (1998) HIV-1 selection by epidermal dendritic cells during transmission across human skin. Journal of Experimental Medicine 187, 1623-1631.

Reis e Sousa, C. (1997) In vivo microbial stimulation induces rapid CD40L-independent production of IL-12 by dendritic cells and their re-distribution to T-cell areas. Journal of Experimental Medicine 186, 1819-1829.

Reis e Sousa, C., Stahl, P.D. and Austyn, J.M. (1993) Phagocytosis of antigens by Langerhans cells *in vitro*. Journal of Experimental Medicine 178, 509-519.

Rescigno, M. and Borrow, P. (2001) The host-pathogen interaction: New themes from dendritic cell biology. Cell 106, 267-270.

Robinson, M.M., Cheevers, W.P. and Burger, D. (1990) Organ-specific modifications of the dose-response relationship of scrapie infectivity. Journal of Infectious Diseases 161, 783-786.

Roitt, I. (1994) Essential Immunology. 8th ed, 1. Blackwell Science, London.

Romani, N., Lenz, A., Glassel, H., Stossel, H., Stanzl, U., Majdic, O., Fritsch, P. and Schuler, G. (1989) Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. Journal of Investigative Dermatology 93, 600-608.

Rose, M.L. (1980) Peanut lectin binding properties of germinal centres of mouse lymphoid centres. Nature 284, 364-366.

Roucou, X., Gains, M. and LeBlanc, A.C. (2004) Neuroprotective functions of prion protein. Journal of Neuroscience Research 75, 153-161.

Rudyk, H., Vasiljevic, S., Hennion, R.M., Birkett, C.R., Hope, J. and Gilbert, I.H. (2000) Screening congo red and its analogues for their ability to prevent the formation of PrP-res in scrapie-infected cells. Journal of General Virology 81, 1155-1164.

Ryan, S., Tiley, L., McConnell, I. and Blacklaws, B. (2000) Infection of dendritic cells by the Maedi-Visna Lentivirus. Journal of Virology 74, 10096-10103.

Saeki, H., Moore, A.M., Brown, M.J. and Hwang, S.T. (1999) Secondary lymphoid tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. Journal of Immunology 162, 2472-2475.

Saeki, H., Wu, M.T., Olasz, E. and Hwang, S.T. (2000) A migratory population of skin derived dendritic cells expresses CXCR5, responds to B-lymphocyte chemoattractant in vitro and co-localizes to B cell zones in lymph nodes *in vivo*. European Journal of Immunology 30, 2808-2814.

Sagebiel, R.W. and Odland, G.F. (1972) Ultrastructural identification of melanocytes in early human embryos. In: V. Riely (Ed), Pigmentation, its genetics and biologic control., pp. 43-50. Appleton-Century-Crofts, New York.

Sallusto, F. and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate antigen to the MHC class II compartment. Downregulation by cytokines and bacterial products. Journal of Experimental Medicine 182, 389-400.

Santambrogio, L., Sato, A.K., Carven, G.J., Belyanskaya, S.L., Strominger, J.L. and Stern, L.J. (1999) Extracellular antigen procesing and presentation by immature dendritic cells. Proceedings of the National Academy of Sciences of the United States of America 96, 15056-15061.

Sarobe, P., Lasarte, J.J., Zabaleta, A., Arribillaga, L., Arina, A., Melero, I., Borras-Cuesta, F. and Prieto, J. (2003) Hepatitis C virus structural proteins impair dendritic cell maturation and inhibit *in vivo* induction of cellular immune responses. Journal of Virology 77, 10862-10871.

Schuhmachers, G., Xu, S., Bergstresser, P.R. and Takashima, A. (1995) Identity and functional properties of novel skin-derived fibroblast lines (NS Series) that support the growth of epidermal-derived dendritic cell lines. Journal of Investigative Dermatology 105, 225-230.

Schuler, G. and Steinman, R.M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells *in vitro*. Journal of Experimental Medicine 161, 526-546.

Schulz-Schaeffer, W.J., Tschoke, S., Kranefuss, N., Drose, W., Hause-Reitner, D., Giese, A., Groschup, M.H. and Kretzschmar, H.A. (2000) The Paraffin-Embedded Tissue Blot detects PrP^{Sc} early in the incubation time in prion diseases. American Journal of Pathology 156, 51-56.

Schwarz, A.O., Kratke, M., Burwinkel, M., Riemer, C., Schultz, J., Henklein, P., Bamme, T. and Baier, M. (2003) Immunisation with a synthetic prion protein-derived peptide prolongs survival times of mice orally exposed to the scrapie agent. Neuroscience Letters 350, 187.

Scott, J.R. (1993) Scrapie pathogenesis. In: I.V. Allen (Ed), Spongiform Encephalopathies, pp. 778-791. Vol. 49. Churchill Livingstone.

Sethi, S., Lipford, G., Wagner, H. and Kretzschmar, H. (2002) Postexposure prophylaxis against prion diseases with a stimulator of innate immunity. Lancet 360, 9328-9331.

Shimada, S., Caughman, S.W., Sharrow, S.O., Stephany, D. and Katz, S.I. (1987) Enhanced antigen-presenting capacity of cultured Langerhans cells is associated with markedly increased expression of Ia antigen. The Journal of Immunolgy. 139, 2551-2558.

Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F. and Stall, A.M. (1992) RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 68, 855-867.

Sigurdson, C.J., Barillas-Mury, C., Miller, M.W., Oesch, B., van Keulen, L.J., Langeveld, J.P. and Hoover, E.A. (2002) PrP (CWD) lymphoid cell targets in early and advanced chronic wasting disease of mule deer. Journal of General Virology 83, 2617-2628.

Sigurdson, C.J., Williams, E.S., Miller, M.W., Spraker, T.R., O'Rourke, K.I. and Hoover, E.A. (1999) Oral transmission and early lymphoid tropism of chronic wasting disease PrP^{res} in mule deer (*Odocolieus hemionus*). Journal of General Virology 80, 2757-2764.

Sigurdsson, E.M., Sy, M.-S., Li, R., Scholtzova, H., Kascsak, R.J., Kascsak, R.J., Carp, R., Meeker, H.C., Frangione, B. and Wisniewski, T. (2003) Anti-prion antibodes for prophylaxis following prion exposure in mice. Neuroscience Letters 336, 185-187.

Silberberg-Sinakin, I., Thorbecke, G.J., Baer, R.I., Rosenthal, S.A. and Berezowsky, V. (1976) Antigen bearing Langerhans cells in skin, dermal lymphatics and in lymph nodes. Cell Immunology 25, 137-151.

Solforosi, L., Criado, J.R., McGavern, D.B., Wirz, S., Sanchez-Alavez, M., Sugama, S., DeGiorgio, L.A., Volpe, B.T., Wiseman, E., Abalos, G., Masliah, E., Gilden, D., Oldstone, M.B.A., Conti, B. and Williamson, R.A. (2004) Cross-linking of celllular prion protein triggers neuronal apoptosis *in vivo*. Science 303, 1514-1516.

Somerville, R.A. (1999) Host and transmissible spongiform encephalopathy agent strain control glycosylation of PrP. Journal of General Virology 80, 1865-1872.

Somerville, R.A., Birkett, C.R., Farquhar, C.F., Hunter, N., Glodmann, W., Dornan, J., Grover, D., Hennion, R.M., Percy, C., Foster, J. and Jeffrey, M. (1997a) Immunodetection of PrP^{Sc} in spleens of some scrapie-infected sheep but not BSE-infected cows. Journal of General Virology 78, 2389-2396.

Somerville, R.A., Chong, A., Mulqueen, O., Birkett, C.R., Wood, S.C.E.R. and Hope, J. (1997b) Biochemical typing of scrapie strains. Nature 386, 564.
Somerville, R.A. and Dunn, A.J. (1996) The association between PrP and infectivity in scrapie and BSE-infected mouse brain. Archives of Virology 141, 275-289.
Somerville, R.A., Oberthur, R.C., Havekost, U., MacDonald, F., Taylor, D.M. and Dickinson, A.G. (2002) Characteristics of thermodynamic diversity between TSE agent stains. Journal of Biological Chemistry 277, 11084-11089.
Song, W.-C., Sarrias, M.R. and Lambris, J.D. (2000) Complement and innate immunity. Immunopharmocology 49, 187-198.
Soong, L., Xu, J.C., Grewal, I.S., Kima, P., Sun, J., Longley Jr, B.J., Ruddle, N.H., McMahon-Pratt, D. and Flavell, R.A. (1996) Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. Immunity 4, 263-273.
Soto, C. (1999) Alzheimers and prion diseases as disorders of protein conformation: implications for the design of novel therapeutic approaches. Journal of Molecular Medicine 77, 412-418.
Soto, C., Kascsak, R.J. and Saborio, G.P. (2000) Reversion of prion protein conformational changes by synthetic β-sheet breaker peptides. Lancet 355, 192-197.
Stahl, N., Baldwin, M.A., Teplow, D.B., Hood, L.E., Gibson, B.W. and Burlingame, A.L. (1993) Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. Biochemistry 32, 1991-2002.
Steinman, R.M., Lustig, D.S. and Cohn, Z.A. (1974) Identification of a novel cell type in perpherial lymphoid organs of mice III : Function properties *in vitro.* Journal of Experimental Medicine 139, 1431-1445.
Steinman, R.M. (1991) The dendritic cell system and its role in immunogenicity. Annual Reviews of Immunology 9, 271-296.
Stoitzner, P., Zanella, M., Ortner, U., Kukas, M., Tagwerker, A., Janke, K., Lutz, M.B., Schuler, G., Echtenacher, B., Ryffel, B., Koch, F. and Romani, N. (1999) Migration of Langerhans cells and dermal dendritic cells in skin organ cultures: augmentation by TNF-alpha and IL-1 beta. Journal of Leukocyte Biology 66, 462-470.
Streilein, J.W. (1989) Antigen-presenting cells in the induction of contact hypersensitivity in mice: evidence that Langerhans cells are sufficient but not required. Journal of Investigative Dermatology 93, 433-448.
Strunk, D., Egger, C., Leitner, G., Hanau, D. and Stingl, G. (1997) A skin homing molecule defines the Langerhans cell progenitor in human peripheral blood. Journal of Experimental Medicine 185, 1131-1136.
Sugaya, M., Nakamura, K., Watanabe, T., Asahina, A., Yasaka, N., Koyama, Y. and al, e. (2002) Expression of cellular prion-related protein by murine Langerhans cells and keratinocytes. Journal of Dermatological Science 28, 126-134.
Tagliavini, F., Forloni, G. and Colombo, L. (1997) Effectiveness of anthracycline against experimental prion disease in Syrian hamster. Science 276, 1119-1122.
Tagliavini, F., Forloni, G. and Colombo, L. (2000) Teracycline affects abnormal properties of synthetic peptides and PrP^{Sc} *in vitro*. Journal of Molecular Biology 300, 1309-1322.

Takashima, A., Edelbaum, D., Kitajima, T., Shadduck, R.K., Gilmore, G.L., Xu, S., Taylor, R.S., Bergstresser, P.R. and Ariizumi, K. (1995) Colony-stimulating factor-1 secreated by fibroblasts promotes the growth of dendritic cell lines (XS series) derived from murine epidermis. Journal of Immunology 154, 5128-5135.

Takeuchi, J., Watari, E., Shinya, E., Norose, Y., Matsumoto, M., Seya, T., Sugita, M., Kawana, S. and Takahashi, H. (2003) Down-regulation of Toll-like receptor expression in monocyte derived Langerhans cell-like cells: implications of low-responsiveness to bacterial components in the epidermal Langerhans cells. Biochemical and Biophysical Research Communications 306, 674-679.

Tal, Y., Souan, L., Cohen, I.R., Meiner, Z., Taraboulos, A.T. and Mor, F. (2003) Complete freund's adjuvant immunization prolongs survival in experimental prion disease in mice. Journal of Neuroscience Research 71, 286-290.

Tan, J., Town, T., Mori, T., Obregon, D., Wu, Y., DelleDonne, A., Rojiani, A., Crawford, F., Flavell, R.A. and Mullan, M. (2002) CD40 is expressed and functional on neuronal cells. EMBO Journal 21, 643-652.

Taylor, D.M., Mc Connell, I. and Fraser, H. (1996a) Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. Journal of General Virology 77, 1595-1599.

Taylor, D.M., McConnell, I. and Fernie, K. (1996b) The effect of dry heat on the ME7 stain of mouse-passaged scrapie agent. Journal of General Virology 77, 3161-3164.

Taylor, P.R. (1998) A targetted disruption of the murine complement factor B gene resulting in the loss of expression of three genes in close proximity, factor B, C2 and D17H6S45. Journal of Biological Chemistry 273, 1699-1704.

Taylor, P.R., Pickering, M.C., Kosco-Vilbois, M.H., Walport, M.J., Botto, M., Gordon, S. and Martinez-Pomares, L. (2002) The follicular dendritic cell restricted epitope, FDC-M2, is complement C4; localisation of immune complexes in mouse tissue. European Journal of Immunology 32, 1888-1896.

Tew, J.G., Kapasi, Z., Qin, D., Wu, J., Haley, S.T. and Szakal, A.K. (1999) Origin of follicular dendritic cells. In: M.T. Lotze and A.W. Thomson (Eds), Dendritic Cells Biology and Clinical Applications, pp. 51-62. Academic Press.

Tew, J.G., Kosco, M.H., Burton, G.F. and Szakal, A.K. (1990) Follicular dendritic cells as accessory cells. Immunological Reviews 117, 185-211.

Tew, J.G., Wu, J., Qin, D., Helm, S., Burton, G.F. and Szakal, A.K. (1997) Follicular dendritic cells and presentation of antigen and costimulatory signals to B-cells. Immunology Reviews 156, 39-52.

Thornberry, N.A. (1991) Interleukin-1β converting enzyme. Methods in Enzymology 244, 615-631.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Tocci, M.J. and *al, e.* (1992) A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. Nature 356, 768-774.

Thornberry, N.A., Peterson, E.P., Zhao, J.J., Howard, A.D. and Griffin, P.R. (1994) Inactivation of interleukin-1 beta converting enzyme by peptide (acyloxy)methyl ketones. Biochemistry 33, 3934-.

Timares, L., Takashima, A. and Johnston, S.A. (1998) Quantitative analysis of the immunopotency of genetically transfected dendritic cells. Proceedings of the

National Academy of Sciences of the United States of America 95, 13147-13152.

Tkachuk, M., Bolliger, S., Ryffel, B., Pluschke, G., Banks, T.A., Herren, S., Gisler, R.H. and Kosco-Vilbois, M.H. (1998) Crucial role of tumour necrosis factor receptor 1 expression on non-hematopoietic cells for B-cell localization within the splenic white pulp. Journal of Experimental Medicine 187, 469-477.

Tobler, I., Gaus, S.E., Deboer, T., Achermann, P., Fischer, M. and al, e. (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. Nature 380, 639-642.

Valladeau, J., Clair-Moninot, V., Dezutter-Dambuyant, C., Pin, J.J., Kissenpfennig, A., Mattei, M.G., Ait-Yahia, S., Bates, E.E., Malissen, B., Koch, I., Fossiez, F., Romani, N., Lebecque, S. and Saeland, S. (2002) identification of mouse langerin/CD207 in Langerhans cells and some dendritic cells of lymphoid tissues. Journal of Immunology 15, 782-792.

Van De Berg, T.K., Yoshida, K. and Dukstra, C.D. (1995) Mechanisms of immune complex trapping by follicular dendritic cells. Current Topics in Microbiology and Immunology 201, 49-63.

van Keulen, L.J.M., Schreuder, B.E.C., Meloen, R.H., Mooij-Harkes, G., Vromans, M.E.W. and Langeveld, J.P.M. (1996) Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. Journal of Clinical Microbiology 34, 1228-1231.

von Andrian, U.H. and Mempel, T.R. (2003) Homing and cellular traffic in lymph nodes. Nature Reviews Immunology 3, 867-878.

von Stebut, E., Belkaid, Y., Jakob, T., Sacks, D.L. and Udey, M.C. (1998) Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells:Implications for the intiation of anti-Leishmania immunity. Journal of Experimental Medicine 188, 1547-1552.

Wang, B., Kondo, S., Shivji, G.M., Fujisawa, H., Mak, T.W. and Sauder, D.N. (1996) Tumour necrosis factor receptor II (p75) signaling is required for the migration of Langerhans cells. Immunology 88, 284-288.

Wang, W., Fujisawa, H. and Zhuang, L. (1997) Depressed Langerhans cell migration and reduced contact hypersensitivity responses in mice lacking TNF recptor p75. Journal of Immunology 159, 6148-6155.

Ware, C.F., VanArsdale, T.L., Crowe, P.D. and Browning, J.L. (1995) The ligands and receptors of the lymphotoxin system. Current Topics in Microbiology and Immunology 198, 175-218.

Wells, G.A.H., Scott, A.C., Johnston, C.T., Gunning, R.F. and Hancock, R. (1987) A novel progressive spongiform encephalopathy in cattle. Veterinary Records 31, 419-420.

Westway, D. and Prusiner, S.B. (1986) Conservation of the cellular gene encoding the scrapie prion protein. Nucleic Acids Research 14, 2035-2044.

White, A.R., Enever, P., Tayebi, M., Mushens, R., Linehan, J., Brandner, S., Anstee, D., Collinge, J. and Hawke, S. (2003) Monoclonal antibodies inhibit prion replication and delay the development of prion disease. Nature 422, 80-83.

Wilesmith, J.W., Ryan, J.B.M. and Atkinson, M.J. (1991) Bovine spongiform encephalopathy: epidemiological studies on the origin. Veterinary Record 128, 199-203.

Will, R.G., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A. and Smith, P.G. (1996) A new varient of Creutzfeldt-Jakob disease in the U.K. Lancet 347, 921-925.

Williams, A., Lucassen, P.J., Ritchie, D. and Bruce, M.E. (1997) PrP deposition and microglial activation and neuronal apoptosis in murine scrapie. Experimental Nephrology 144, 433-438.

Williams, E.S. and Young, S. (1980) Chronic wasting disease of captive mule deer: a spongiform encephalopathy. Journal Wildlife Diseases 16, 89-98.

Williams, P.L. (1995) Integumental system. In: L.H. Bannister, M.M. Berry, P. Collons, M. Dyson, J.E. Dussek and M.W.J. Ferguson (Eds), Gray's Anatomy, 38th ed., pp. 376-416. Vol. 1. Churchill Livingstone.

Winkelmann, R.K. (1973) The merkel cell. Journal of Investigative Dermatology 60, 2-15.

Wisniewski, H.M., Sigudarson, S., Rubenstein, R., Kascsak, R.J. and Carp, R.I. (1996) Mites as vectors for scrapie. Lancet 347, 1114.

Wong, B.-S., Liu, R.-L., Pan, T., Petersen, R.B., Smith, M.A., Gambetti, P., Perry, G., Manson, J.C., Brown, D.R. and Sy, M.-S. (2001) Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein. Journal of Neurochemistry 76, 565-572.

Worrell, J.T. and Cockerell, C.J. (1997) Histopathology of peripheral nerves in cutaneous herpesvirus infection. American Journal of Dermatopathology 19, 133-137.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J. and Mathison, J.C. (1990) CD14 a receptor for complexes of lipopolysaccharide(LPS) and LPS binding protein. Science 249, 1431-1433.

Wu, Q., Wang, Y., Hedgeman, E.O., Browning, J.L. and Fu, Y.-X. (1999) The requirement of membrane lymphotoxin for the presence of dendrtic cells in lymphoid tissues. Journal of Experimental Medicine 190, 629-638.

Wu, S.-J.L., Grouard-Vogel, G., Sun, W., Mascola, J.R., Brachtel, E., Putvatana, R., Louder, M.K., Filgueira, L., Marovich, M.A., Wong, H.K., Blauvelt, A., Murphy, G.S., Robb, M.L., Innes, B.L., Birx, D.L., Hayes, C.G. and Schlesinger-Frankel, S. (2000) Human skin Langerhans cells are targets for dengue virus infection. Nature Medicine 6, 816-820.

Wyatt, J.M., Pearson, G.R., Smerdon, T.N., Gruffydd-Jones, T.J., Wells, G.A.H. and Wilesmith, J.W. (1991) Naturally occurring scrapie-like spongiform encephalopathy in five domestic cats. Veterinary Record 129, 233-236.

Wykes, M., Pombo, A., Jenkins, C. and MacPherson, G. (1998) Dendritic cells interact directly with naive B-lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. Journal of Immunology 161, 1313-1319.

Xu, H., Ariizumi, K., Caceres-Dittmar, G., Edelbaum, D., Hashimoto, K., Bergstresser, P.R. and Takashima, A. (1995a) Successive generation of antigen-presenting dendritic cell lines from murine epidermis. Journal of Immunology 154, 2697-2705.

Xu, H., Bergstresser, P.R. and Takashima, A. (1995b) Phenotypic and functional heterogeneity among murine epidermal-derived dendritic cell clones. Journal of Investigative Dermatology 105, 831-836.

Xu, J., Foy, T.M., Laman, J.D., Elliott, E.A., Dunn, J.J., Waldschmidt, T.J., Elsemore, J., Noelle, R.J. and Flavell, R.A. (1994) Mice Deficient for the CD40 Ligand. Immunity 1, 423-431.

Yamada, N. and Katz, S.I. (1999) Generation of mature dendritic cells from CD14+ cell line (XS52) by IL-4, TNF-alpha, IL-1 beta, and agonistic anti-CD40 monoclonal antibody. Journal of Immunology 163, 5331-5337.

Yoshino, M., Yamazaki, H., Nakano, H., Kakiuchi, T., Ryoke, K., Kunisada, T. and Hayashi, S.-I. (2003) Distinct antigen trafficking from the skin in steady and active states. International Immunology 15, 773-779.

Yu, P., Wang, Y., Chin, R.K., Martinez-Pomares, L., Gordon, S., Kosco-Vilbois, M.H., Cyster, J. and Fu, Y.-X. (2002) B-cells control the migration of a subset of dendritic cells into B-cell follicules via CXC chemokine ligand 13 in a lymphotoxin-dependent fashion. Journal of Immunology 168, 5117-5123.

Zanusso, G., Liu, D., Ferrari, S., Hegyi, I., Yin, X., Aguzzi, A., Hornemann, S., Liemann, S., Glockshiber, R., Manson, J.C., Brown, P., Petersen, R.B., Gambetti, P. and Sy, M.S. (1998) Prion protein expression in different species: analysis with a panel of new mAbs. Proceedings of the National Academy of Sciences of the United States of America 95, 8812-8816.

Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Riccia-Castagnoli, P., Raposo, G. and Amigorena, S. (1998) Eradication of established murine tumors using a novel cell-free vaccine: dendrtic cell-derived exosomes. Nature Medicine 4, 594-600.

Zlotnik, I. and Rennie, J.C. (1963) Further observations on the experimental transmission of srapie from sheep and goats to laboratory mice. The Journal of Comparative Pathology and Therapeutics. 73, 150-163.

Journal of Dermatological Science (2004) 35, 101–111

ELSEVIER

Journal of Dermatological SCIENCE

www.intl.elsevierhealth.com/journals/jods

Scrapie transmission following exposure through the skin is dependent on follicular dendritic cells in lymphoid tissues

Joanne Mohan, Karen L. Brown, Christine F. Farquhar, Moira E. Bruce, Neil A. Mabbott*

Institute for Animal Health, Ogston Building, West Mains Road, Edinburgh EH9 3JF, UK

Received 9 March 2004; received in revised form 22 April 2004; accepted 12 May 2004

KEYWORDS
Transmissible spongiform encephalopathy;
Scrapie;
Skin;
Follicular dendritic cell;
Prion protein;
Spleen

Summary ***Background:*** Transmissible spongiform encephalopathies (TSEs) are chronic infectious neurodegenerative diseases that are characterized by the accumulation in affected tissues of PrP^{Sc}, an abnormal isoform of the host prion protein (PrP^{C}). Following peripheral exposure, PrP^{Sc} usually accumulates on follicular dendritic cells (FDCS) in lymphoid tissues before neuroinvasion. Studies in mice have shown that TSE exposure through scarified skin is an effective means of transmission. Following inoculation via the skin, a functional immune system is critical for the transmission of scrapie to the brain as severe combined immunodeficiency (SCID) mice are refractory to infection. Until now, it was not known which components of the immune system are required for efficient scrapie neuroinvasion following skin scarification. ***Objective:*** To determine which cells are critical for the transmission of scrapie to the brain following inoculation via the skin. ***Methods:*** A chimeric mouse model was used, which had a mismatch in PrP^{C} expression between FDCs and other bone marrow-derived cells within lymphoid tissues. These chimeric mice were challenged with scrapie by skin scarification to allow the separate roles of FDCs and lymphocytes in peripheral scrapie pathogenesis to be determined. ***Results:*** We show that mature FDCs are essential for the accumulation of scrapie within lymphoid tissues and the subsequent transmission of infection to the brain following TSE exposure by this route. Furthermore, we show that the accumulation of PrP^{Sc} and infectivity in the spleen is independent of PrP expression by lymphocytes or other bone marrow-derived cells. ***Conclusion:*** Following inoculation with scrapie by skin scarification, replication in the spleen and subsequent neuroinvasion is critically dependent upon mature FDCs.

1. Introduction

The transmissible spongiform encephalophathies (TSEs or prion diseases) are a group of infectious, fatal, neurodegenerative diseases, which affect both animals and humans. The precise nature of the TSE agent is still subject to debate [1]. However, PrP^{Sc}, an abnormal, detergent-insoluble, relatively proteinase-resistant isoform of a host glycoprotein PrP^{C} [2], is considered to constitute a major or possibly the sole component of the infectious agent [3]. The deposition of PrP^{Sc} within the brain of an infected

*Corresponding author. Tel.: +44 131 667 5204; fax: +44 131 668 3872.
E-mail address: neil.mabbott@bbsrc.ac.uk (N.A. Mabbott).

0923-1811/$30.00
doi:10.1016/j.jdermsci.2004.05.005

host correlates in most TSE diseases with the development of neuropathological changes, such as vacuolation, gliosis, and neuronal loss.

Many TSEs including natural sheep scrapie, bovine spongiform encephalopathy (BSE), chronic wasting disease in mule deer and elk, and variant Creutzfeldt-Jakob disease (vCJD) in humans are thought to be acquired by peripheral exposure. For example, the consumption of BSE-contaminated meat products by humans is most likely to be responsible for the emergence of vCJD [4]. Following peripheral inoculation with TSE agents, high levels of infectivity and PrP^{Sc} usually accumulate in lymphoid tissues prior to the dissemination of infection to the central nervous system (CNS). Within the lymphoid tissues of TSE-infected hosts [5–10], PrP^{Sc} accumulation initially takes place in germinal centres in association with follicular dendritic cells (FDCs). Studies in rodents, inoculated intra-peritoneally with scrapie, have shown that mature FDCs are critical for scrapie accumulation in lymphoid tissues. Furthermore, in the absence of mature FDCs, the spread of disease to the CNS is significantly impaired [7,8,11–14]. From the lymphoid tissues, infectivity is translocated to the CNS via peripheral nerves [15].

Although oral acquisition (ingestion) is considered to be the main route of natural exposure to TSE agents, other potential routes of exposure have been identified. Sporadic sCJD in humans has been transmitted iatrogenically through transplantation of sCJD-contaminated tissues or pituitary-derived hormones [16]. BSE and natural scrapie have also been shown to be transmissible experimentally by blood transfusion between sheep [17,18] raising speculation that vCJD in humans might also be transmitted via blood transfusion from an infected donor. Studies in mice have shown that skin scarification is an effective means of scrapie transmission highlighting another possible route of exposure [19]. For example, some cases of natural sheep scrapie might be transmitted through sites of skin scarification or skin lesions during close contact with other scrapie-infected animals [20]. Scrapie might also be established through skin or gum lesions in the mouth [21], or be passed from mother to offspring via the unhealed umbilical cord or through sites of skin trauma at birth. Surgical instruments contaminated with sCJD infectivity have also been shown to have the potential to transmit disease [22]. Animal models of TSE transmission through scarified skin highlight important health and safety issues, which include whether scientists and health workers are at risk of acquiring infectivity when handling infected tissues or instruments. Biopharmaceutical and cosmetic products derived from sheep and cattle tissues might harbour TSE infectivity, with the potential to transmit disease when applied to abraded skin [23,24]. Understanding the immunobiology of scrapie transmission via the skin will help in determining the possible significance of this route in natural TSE infections and aid the development of therapeutic strategies.

Previous studies have shown that a functional immune system is critical for the translocation of scrapie to the CNS following skin scarification, as severe combined immunodeficiency (SCID) mice are refractory to scrapie infection by this route [19]. Studies in this laboratory using the ME7 scrapie strain have shown that following inoculation by intra-peritoneal injection, mature FDCs are critical for efficient neuroinvasion [7,8,13,44,45]. However, whether FDCs or other components of the immune system are required for efficient scrapie neuroinvasion following inoculation by skin scarification is not known. For example, as the skin is highly innervated, it is plausible that lymphocytes or Langerhans cells acquire scrapie within the skin and transport it directly to peripheral nerves. To maintain TSE infection, host cells must express the cellular isomer of the host prion protein, as mice deficient in PrP^{c} ($Prnp^{-/-}$ mice) do not develop disease [25,26]. Thus, in order to determine which cells are critical for the efficient transmission of scrapie to the CNS following inoculation via the skin, we used a chimeric mouse model previously established in this laboratory, which had a mismatch in PrP^{c} status between FDCs and other bone marrow-derived cells within lymphoid tissues [7]. These chimeric mice were challenged with scrapie by skin scarification to allow the separate roles of FDCs and lymphocytes in peripheral scrapie pathogenesis to be determined.

2. Material and methods

2.1. Mice and bone marrow grafting

129/*Ola* mice were used as immunocompetent wild-type controls. Bone marrow (BM) from the femurs and tibias of immunocompetent adult 129/*Ola* ($Prnp^{+/+}$) mice or PrP-deficient 129/*Ola* ($Prnp^{-/-}$) mice [26] was prepared as a single-cell suspension (3×10^7 to 4×10^7 viable cells per ml) in Hank's balanced salt solution (Life Technologies, Paisley, UK). Recipient SCID/$Prnp^{+/+}$ mice [7] were reconstituted with 0.1 ml BM by injection into the tail vein. Recipient mice and age-matched ungrafted controls were used in subsequent experiments 28 days after BM grafting. All mice were housed in individually ventilated cages to ensure

a high standard of microbiological hygiene. All protocols using experimental rodents were approved by the Institute's Protocols and Ethics Committee and carried out according to the strict regulations of the UK Home Office 'Animals (scientific procedures) Act 1986'.

2.2. Scrapie challenge

Mice were inoculated with the ME7 scrapie strain by skin scarification of the medial surface of the left thigh. Briefly, prior to scarification, approximately 1 cm^2 area of hair covering the site of scarification was trimmed using curved scissors and then removed completely with an electric razor. Twenty-four hours later a 23-gauge needle was used to create a 5 mm long abrasion in the epidermal layers of the skin at the scarification site. Then using a 26-gauge needle, one droplet (~6 μl) of ME7 scrapie inoculum from a 1% (w/v) terminal scrapie mouse brain homogenate in physiological saline was applied to the abrasion and worked into the site using sweeping strokes. The scarification site was then sealed with OpSite (Smith and Nephew Medical Ltd., Hull, UK) and allowed to dry before the animals returned to their final holding cages. Where indicated separate groups of mice were inoculated by intra-cerebral (i.c.) injection with 20 μl of the same 1% scrapie mouse brain homogenate in physiological saline (a dose of approximately $1 \times 10^{4.5}$ i.c. 50% infectious dose [ID_{50}] units) as a titre control. Following challenge, animals were coded, assessed weekly for signs of clinical disease, and killed at a standard clinical end-point [27]. Scrapie diagnosis was confirmed by histopathological assessment of TSE vacuolation in the brain. For bioassay of scrapie infectivity, half spleens were pooled from four animals from each group and prepared as 10% (w/v) homogenates in physiological saline and 20 μl injected i.c. into groups of 12 C57BL indicator mice. The scrapie titer in each spleen was determined from the mean incubation period in the assay mice, by reference to established dose/incubation period response curves for scrapie-infected spleen tissue [28].

2.3. Immunohistochemical analysis

To monitor FDC status, spleen halves were snap-frozen and stored at the temperature of liquid nitrogen. Serial frozen sections (thickness, 10 μm) were cut on a cryostat and FDCs were visualized by staining with the FDC-specific rat monoclonal antiserum FDC-M2 (AMS Biotechnology, Abingdon, UK), or 8C12 monoclonal antiserum to detect CD35 (BD PharMingen, Oxford, UK). B-lymphocytes were also detected using the rat monoclonal antiserum B220 to detect CD45R (Caltag, Towcester, UK). Immunolabelling was carried out using alkaline phosphatase coupled to the avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA). Vector Red (Vector Laboratories) was used as a substrate.

For the detection of PrP in brain tissue, tissues were fixed in periodate-lysine-paraformaldehyde and embedded in paraffin wax. Sections (thickness, 6 μm) were deparaffinized, and pretreated to enhance PrP immunostaining by hydrated autoclaving (15 min, 121 °C), and subsequent immersion in formic acid (98%) for 5 min [5]. Sections were then stained with the PrP-specific monoclonal antiserum 6H4 (Prionics, Zürich, Switzerland) and immunolabelling detected using hydrogen peroxidase coupled to the avidin–biotin complex (Vector Laboratories) with diaminobenzidine (DAB) as a substrate.

All sections were counterstained with hematoxylin to distinguish cell nuclei.

2.4. *Prnp* genotype analysis of spleen tissue

Total DNA was extracted from spleen tissue fragments (approximately 5 mg) by proteinase K digestion and purified by phenol–chloroform extraction by standard techniques. The *Prnp* genotypes of spleen samples from BM-grafted animals were determined by PCR analysis through the amplification of the *Prnp* gene, and a portion of the neomycin resistance gene to detect the presence of the $Prnp^{-/-}$ genotype [26]. The PCR mixture (total volume 60.7 μl) contained; 5 μl of 10× PCR buffer, 5.0 μl of 50 mm $MgCl_2$, 1.0 μl of 10 mm dNTP mix (Life Technologies), 1 μl of chromosomal DNA, 0.5 μl (100 pmol/μl) of specific primers, 0.2 μl of Taq polymerase (Life Technologies) and 50 μl of sterile Dnase- and Rnase-free water. PCR analysis was performed using the following specfic primers: *Prnp* Fwd-5′-ATG GCG AAC CTT GGC TAC TGG CTG-3′; *Prnp* Rev-5′-TCA TCC CAG GAT CAG CAA GAT GAG-3′. These primers anneal to the start and stop codons of the *Prnp* gene open reading frame, respectively and generate a fragment of 750 bp. The sequences of the oligonucleotide primers for the neomycin resistance gene were: *Neo* Fwd-5′-TTG AGC CTG GCG AAC AGT TC-3′; *Neo* Rev-5′-GAT GGA TTG CAC GCA GGT TC-3′. These primers anneal to the neomycin resistance gene present in $Prnp^{-/-}$ mice, which is located within exon 3 of the *Prnp* gene [26]. These primer pairs were designed to generate a 550 bp fragment.

Following a hot start at 94 °C for 3 min, an amplification cycle was carried out for 30 cycles at the following temperatures: 94 °C for 50 s, 62 °C for 50 s, 72 °C for 50 s on a thermal cycler (Genius

PCR System, Techne, Cambridgeshire, UK). A final extension period at 72 °C for 10 min was included at the end of the 30 cycles. PCR products were resolved by electrophoresis at 125 V through a 1.5% agarose gel containing 1 μg/ml ethidium bromide.

2.5. Immunoblot detection of PrP^{Sc}

Spleen fragments (approximately 20 mg) were prepared as previously described by [29]. Briefly, before immunoblot analysis, tissue homogenates were treated in the presence or absence of 80 μg proteinase K (to confirm the presence of PrP^{Sc}) and subsequently partially purified by treatment with 2% (w/v) *N*-lauroylsarcosine (in 0.1 M Tris–HCl pH 7.4), allowing sedimentation only of the proteinase-K-resistant, detergent-insoluble fraction of PrP (PrP^{Sc}). Samples were subjected to electrophoresis through sodium dodecyl sulphate 10–20% polyacrylamide gels (Bio-Rad, Hemel Hempstead, UK) and transferred to polyvinylidine difluoride membranes (Bio-Rad) by semi-dry blotting. PrP was detected with the PrP-specific mouse monoclonal antiserum 8H4 [30] (a kind gift from Professor Man-Sun Sy, Case Western Reserve University School of Medicine, Cleveland, OH, USA). Immunolabelling was carried out using horseradish peroxidase-conjugated rat anti-mouse antiserum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), and bound horseradish peroxidase activity detected with Supersignal® West Dura Extended Duration Substrate (Pierce, Chester, UK).

2.6. Statistical analysis

All data are presented as the mean ± S.E.M. and error bars are indicated on figures where the S.E.M. was 5% of the mean. The statistical significance of differences in means of experimental groups was calculated using ANOVA one-way analysis with Minitab software. A *P*-value of 0.05 was considered to be significantly different.

3. Results

3.1. Bone marrow reconstitution of SCID/$Prnp^{+/+}$ mice restores scrapie susceptibility following inoculation by skin scarification

To test the hypothesis that mature FDCs are critical for the translocation of scrapie to the CNS following skin scarification, a chimeric mouse model was used, which had a mismatch in PrP status between its FDCs and lymphocyte populations [7]. The mouse models were produced by grafting SCID/$Prnp^{+/+}$ mice with either PrP expressing ($Prnp^{+/+}$) or PrP-deficient ($Prnp^{-/-}$) BM from immunocompetent 129/*Ola* mice. As FDCs are not considered to be derived from BM in adult mice [31,32], the lymphoid tissues of SCID/$Prnp^{+/+}$ mice grafted with $Prnp^{-/-}$ BM (SCID/$Prnp^{+/+}$ + $Prnp^{-/-}$ BM) will have PrP expressing FDCs and other stromal-derived cells but will lack PrP expression on lymphocytes. In contrast, SCID/$Prnp^{+/+}$ mice grafted with $Prnp^{+/+}$ BM (SCID/$Prnp^{+/+}$ + $Prnp^{+/+}$ BM) will contain both PrP expressing FDCs and lymphocytes.

Twenty-eight days after BM grafting, wild-type 129/*Ola* mice, SCID/$Prnp^{+/+}$ mice, SCID/$Prnp^{+/+}$ + $Prnp^{+/+}$ BM mice and SCID/$Prnp^{+/+}$ + $Prnp^{-/-}$ BM mice were inoculated with the ME7 scrapie strain by skin scarification. All immunocompetent wild-type 129/*Ola* mice developed clinical signs of scrapie, approximately 333 ± 13 days post-inoculation ($n = 12$; Fig. 1). Characteristic disease-specific PrP accumulation (Fig. 2a) and spongiform pathology (Fig. 2e) typical of a peripheral infection with the ME7 scrapie strain was detected in the brains of all wild-type mice, which developed clinical disease. In contrast, previous studies have shown that SCID mice are refractory to challenge with ME7 scrapie by skin scarification up to 586 days post-inoculation [19]. Unfortunately, despite careful husbandry, all ungrafted immunodeficient SCID/$Prnp^{+/+}$ mice in this study succumbed to non-infectious, non-TSE diseases (eg: thymic tumours) up to 274 days post-inoculation (Fig. 1). Immunohistochemical analysis of brain tissue from all scrapie-inoculated SCID/$Prnp^{+/+}$ mice failed to detect any signs of disease-specific PrP accumulation (Fig. 2b) or vacuolation (Fig. 2f) consistent with the demonstration that these mice are refractory to scrapie following peripheral inoculation. However, the susceptibility of most SCID/$Prnp^{+/+}$ mice to scrapie infection was restored following grafting with either $Prnp^{+/+}$ or $Prnp^{-/-}$ BM (Fig. 1). Here, 6/11 SCID/$Prnp^{+/+}$ mice grafted with $Prnp^{+/+}$ BM developed scrapie with a mean incubation period of 380 ± 12 days post-inoculation. Likewise 8/10 SCID/$Prnp^{+/+}$ mice grafted with $Prnp^{-/-}$ BM developed scrapie with a mean incubation period of 378 ± 4 days post-inoculation. Characteristic spongiform pathology and disease-specific PrP accumulation were detected in the brains of all grafted SCID/$Prnp^{+/+}$ mice, which succumbed to clinical disease (Fig. 2).

No significant difference was observed between the mean incubation periods of SCID/$Prnp^{+/+}$ mice grafted with either $Prnp^{+/+}$ or $Prnp^{-/-}$ BM. However, a significant statistical difference was

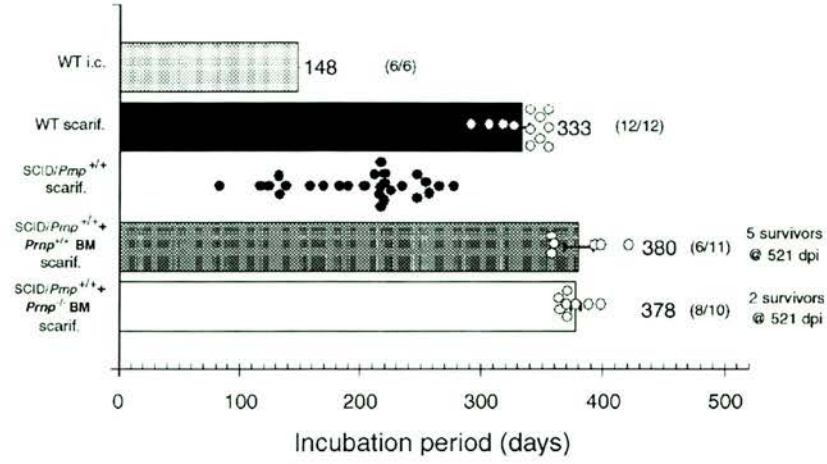


Fig. 1 Reconstitution of SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM restores susceptibility to scrapie when inoculated by skin scarification. Wild-type (WT) mice (■); SCID/*Prnp*$^{+/+}$ mice, SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{+/+}$ BM (□; SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM) and SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{-/-}$ BM (□; SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM) were inoculated with the ME7 scrapie strain by skin scarification (scarif.). Wild-type mice were also inoculated by i.c. injection as a titre control (▩; WT i.c). Each bar represents the mean incubation period ± S.E.M. (○) Incubation periods for individual mice that succumbed to clinical scrapie. (●) Times at which SCID/*Prnp*$^{+/+}$ mice succumbed to non-TSE related disease.

observed between the mean incubation periods of SCID/*Prnp*$^{+/+}$ mice grafted with either *Prnp*$^{+/+}$ BM or *Prnp*$^{-/-}$ BM when compared with wild-type mice ($P = 0.014$ and 0.004, respectively, ANOVA one-way analysis). Grafted SCID/*Prnp*$^{+/+}$ mice developed clinical scrapie approximately 47 days later than the mean incubation period of immunocompetent wild-type controls. However, no significant difference in the pathological targeting of vacuolation in the brain was observed between wild-type and

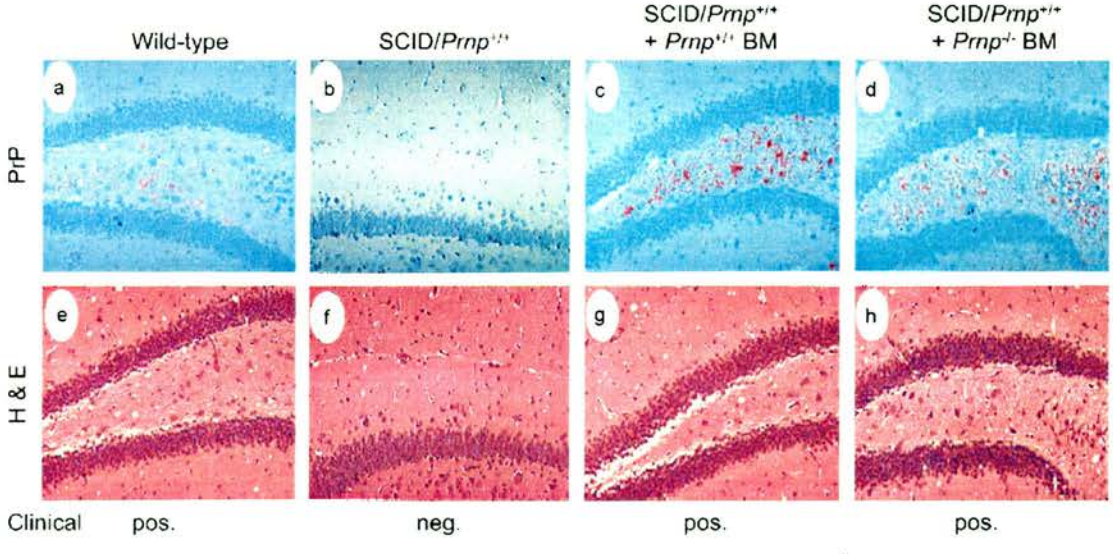


Fig. 2 Histological analysis of brain tissue from wild-type mice (a and e), SCID/*Prnp*$^{+/+}$ mice (b and f), SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{+/+}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM; c and g), and SCID/*Prnp*$^{+/+}$ mice reconstituted with *Prnp*$^{-/-}$ BM (SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM; d and h) inoculated with scrapie by skin scarification. Large PrP accumulations (brown; upper row) and spongiform pathology (H and E; lower row) were detected in the hippocampi of all mice, which developed the clinical signs of scrapie. In contrast, no evidence of PrP accumulation (b) or spongiform pathology (f) was detected in the brains of any SCID/*Prnp*$^{+/+}$ mice that succumbed to non-TSE diseases up to 274 dpi. All sections were counterstained with hematoxylin (blue); pos.: mice that developed clinical signs of scrapie; neg.: mice that were free of the signs of scrapie. Original magnification 200×.

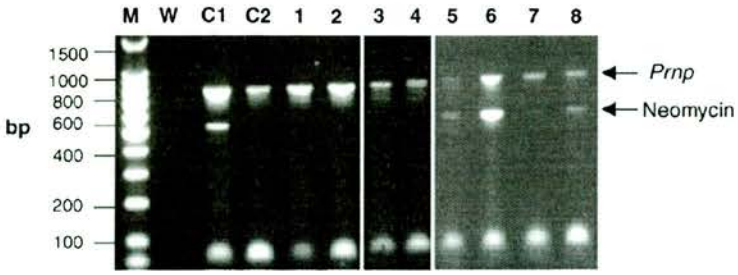


Fig. 3 Confirmation of the *Prnp* genotype in the spleens of SCID/$Prnp^{+/+}$ mice reconstituted with either $Prnp^{+/+}$ BM (lanes 1–4) or $Prnp^{-/-}$ BM (lanes 5–8). Analysis of total splenic DNA from SCID/$Prnp^{+/+}$ mice reconstituted with $Prnp^{+/+}$ BM confirmed the presence only of the *Prnp* gene by the visualisation of a single band at 750 bp (lanes 1–4). The visualisation of two bands at 750 and 550 bp (lanes 5–8) confirmed the presence of both *Prnp* gene and a portion of the neomycin resistance gene (Neomycin), respectively, within splenic DNA samples from SCID/$Prnp^{+/+}$ mice reconstituted with $Prnp^{-/-}$ BM (SCID/$Prnp^{+/+}$ + $Prnp^{-/-}$ BM). Lane M, 200 bp molecular size markers. Controls included: C1, splenic DNA from a SCID/$Prnp^{+/+}$ + $Prnp^{-/-}$ BM mouse; C2, splenic DNA from a wild-type/$Prnp^{+/+}$ mouse; W, PCR-amplified water was used as a negative control.

grafted SCID/$Prnp^{+/+}$ mice suggesting that neuroinvasion had occurred via a common pathway in each case.

Although the susceptibility of most SCID/$Prnp^{+/+}$ mice to scrapie was restored following BM grafting, 5/11 SCID/$Prnp^{+/+}$ + $Prnp^{+/+}$ BM mice and 2/10 SCID/$Prnp^{+/+}$ + $Prnp^{-/-}$ BM mice remained free from the signs of scrapie 521 days post-inoculation, at which point the experiment was terminated (Fig. 1). Successful reconstitution and normal germinal centre architecture were confirmed in these mice (data not shown); however, immunohistochemical analysis of brain tissue from all surviving mice failed to detect any spongiform change or disease-specific PrP accumulation (data not shown). Likewise, no PrP^{Sc} accumulation was detected in the spleen by immunoblot analysis (data not shown) suggesting these mice would not have developed clinical scrapie at a later stage.

3.2. Confirmation of immune status and germinal centre architecture

Spleens and serum were taken from all mice to monitor immune status. Consistent with the absence of B-lymphocytes in SCID mice [33,34], serum from all ungrafted SCID/$Prnp^{+/+}$ mice contained barely detectable levels of immunoglobulin (Ig) when compared with those of wild-type mice (data not shown). However, ELISA analysis confirmed that reconstitution of SCID/$Prnp^{+/+}$ mice with immunocompetent BM from either $Prnp^{+/+}$ or $Prnp^{-/-}$ mice restored serum Ig levels to those observed in wild-type mice (data not shown). Thus functional BM-derived B-lymphocytes had been successfully grafted into recipient SCID/$Prnp^{+/+}$ mice.

We next determined the *Prnp* genotype in the spleens of grafted mice by PCR analysis of total splenic DNA (Fig. 3). Analysis of DNA from SCID/$Prnp^{+/+}$ mice grafted with $Prnp^{+/+}$ BM detected the presence of only the *Prnp* gene by the visualisation of a single band at 750 bp (Fig. 3, lanes 1–4). In contrast, two bands were detected in splenic DNA samples from SCID/$Prnp^{+/+}$ mice grafted with $Prnp^{-/-}$ BM, demonstrating the presence of both the *Prnp* gene (750 bp) and the neomycin resistance gene (550 bp) (Fig. 3, lanes 5–8). Thus, these results confirmed the presence of only $Prnp^{+/+}$ cells within the spleens of SCID/$Prnp^{+/+}$ + $Prnp^{+/+}$ BM mice, and the presence of both $Prnp^{+/+}$ and $Prnp^{-/-}$ cells within the spleens of SCID/$Prnp^{+/+}$ + $Prnp^{-/-}$ BM mice.

The germinal centre architecture in the spleen was analysed by immunohistochemistry. As expected, FDC-M2 and CD35 expressing FDC networks and B-lymphocytes (CD45R/B220) were detected in the spleens of all immunocompetent wild-type mice (Fig. 4). B-lymphocytes produce important factors for the maintenance and maturation of FDCs. In the absence of B-lymphocytes FDCs do not receive these important stimuli and do not mature [33,34]. Thus mice deficient in B-lymphocytes are indirectly deficient in FDCs. As Fig. 4 illustrates, both FDC networks and B-lymphocytes were absent in spleens of ungrafted SCID/$Prnp^{+/+}$ mice, consistent with the immunodeficent phenotype of SCID mice [33,34]. However, FDC networks in the spleens of SCID mice can be restored following grafting with B-lymphocytes or immunocompetent BM as a source of lymphocytes [34]. Likewise, mature FDC networks and B-lymphocytes were restored in spleens of SCID/$Prnp^{+/+}$ mice following grafting with either $Prnp^{+/+}$ or $Prnp^{-/-}$ BM (Fig. 4). Thus, the restoration of germinal center architecture in the lymphoid tissues of SCID/$Prnp^{+/+}$ mice, following BM grafting, coincided with the restored susceptibility of these mice to scrapie.

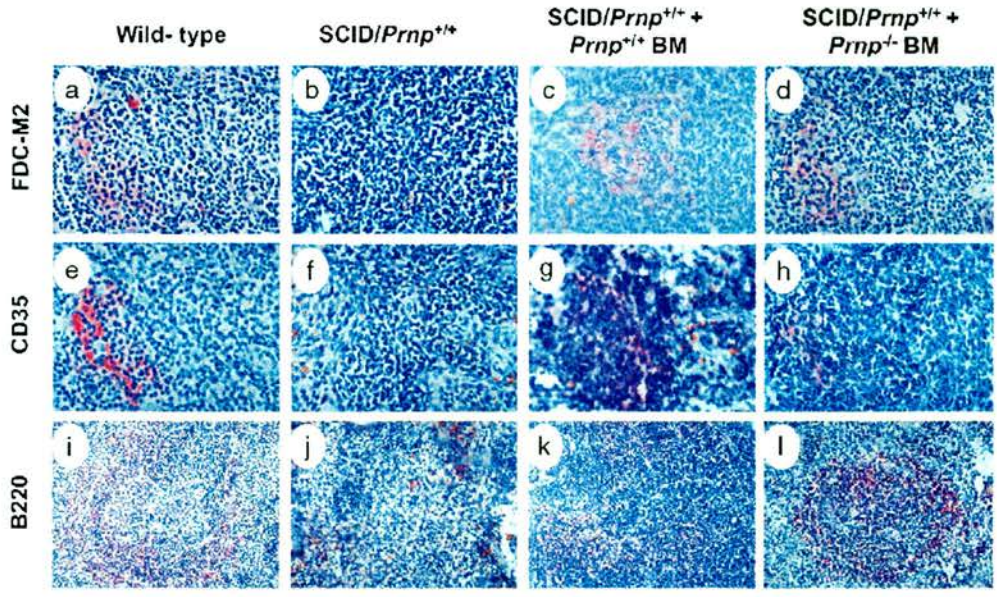


Fig. 4 Immunohistochemical analysis of the germinal centre architecture in spleen tissue from wild-type mice, SCID/$Prnp^{+/+}$ mice, SCID/$Prnp^{+/+}$ mice reconstituted with $Prnp^{+/+}$ bone marrow (SCID/$Prnp^{+/+}$ + $Prnp^{+/+}$ BM), and SCID/$Prnp^{+/+}$ mice reconstituted with $Prnp^{-/-}$ bone marrow (SCID/$Prnp^{+/+}$+ $Prnp^{-/-}$ BM). Adjacent frozen sections were stained with FDC-M2 antiserum to detect FDCs (red; a–d), CD35-specific monoclonal antiserum 8C12 to detect complement receptor 1 (red; e–h) and the CD45R-specific antiserum B220 to detect B-lymphocytes (red; i–l). All sections were counterstained with hematoxylin (blue). As expected, FDC-M2 and CD35 expressing FDC networks and B-lymphocytes (CD45R/B220) were detected in the spleens of all immunocompetent wild-type mice (a, e and i, respectively). In the absence of B-lymphocytes, FDCs do not receive important stimuli and can not mature. Thus mice deficient in B-lymphocytes are indirectly deficient in FDCs. As panels b, f, and j illustrate, both FDC networks and B-lymphocytes were absent in spleens of ungrafted SCID/$Prnp^{+/+}$ mice. However, mature FDC networks and B-lymphocytes were restored in spleens of SCID/$Prnp^{+/+}$ mice following grafting with either $Prnp^{+/+}$ (c, g, and k) or $Prnp^{-/-}$ BM (d, h, and l). Original magnification 400× (a–h), 200× (i–l).

3.3. Scrapie infectivity and PrP^{Sc} accumulation in the spleen

Following peripheral inoculation with the ME7 scrapie strain, high levels of infectivity and the disease-specific isoform of the prion protein, PrP^{Sc}, accumulate in the spleen within 42 days post-inoculation and are maintained throughout the course of infection [29]. Spleen samples were taken from four mice from each experimental group of mice 220 days post-inoculation with scrapie via skin scarification. The scrapie infectivity titre in spleen lysates from each group was estimated by bioassay in groups of 12 indicator mice. As expected, spleens from scrapie-inoculated wild-type mice contained high levels of infectivity (approximately 5.7 log i.c. ID_{50}/g). In contrast, scrapie infectivity was undetectable in spleen samples from ungrafted SCID/$Prnp^{+/+}$ mice assayed 220 days post-inoculation suggesting a scrapie infectivity titre, if present, below 2.6 log i.c. ID_{50}/g (at least 1000-fold less than the level detected in spleens of wild-type mice assayed at the same time post-inoculation). Scrapie infectivity accumulation was restored in the spleens of SCID/$Prnp^{+/+}$ mice following grafting with either $Prnp^{+/+}$ or $Prnp^{-/-}$ BM to the same magnitude observed in wild type mice at the same time point (approximately 6.7 and 5.7 log i.c. ID_{50}/g, for SCID/$Prnp^{+/+}$ mice grafted with either $Prnp^{+/+}$ or $Prnp^{-/-}$ BM, respectively).

Similarly, immunoblot analysis of spleen tissue from terminally affected wild-type mice detected large accumulations of detergent-insoluble proteinase-K-resistant PrP^{Sc} (Fig. 5). A typical three-banded pattern was observed between molecular mass values of 20–30 kDa, representing the unglycosylated, monoglycosylated, and diglycosylated isomers of PrP (in order of increasing molecular mass). However, no PrP^{Sc} accumulation was detectable within the spleens of any ungrafted SCID/$Prnp^{+/+}$ mice assayed at various times after inoculation (Fig. 5a, lanes 4, 6 and 8). In comparison, PrP^{Sc} accumulation was restored in the spleens of terminally scrapie-affected SCID/$Prnp^{+/+}$ mice grafted with either $Prnp^{+/+}$ or $Prnp^{-/-}$ BM to levels similar to those observed in wild type mice (Fig. 5b).

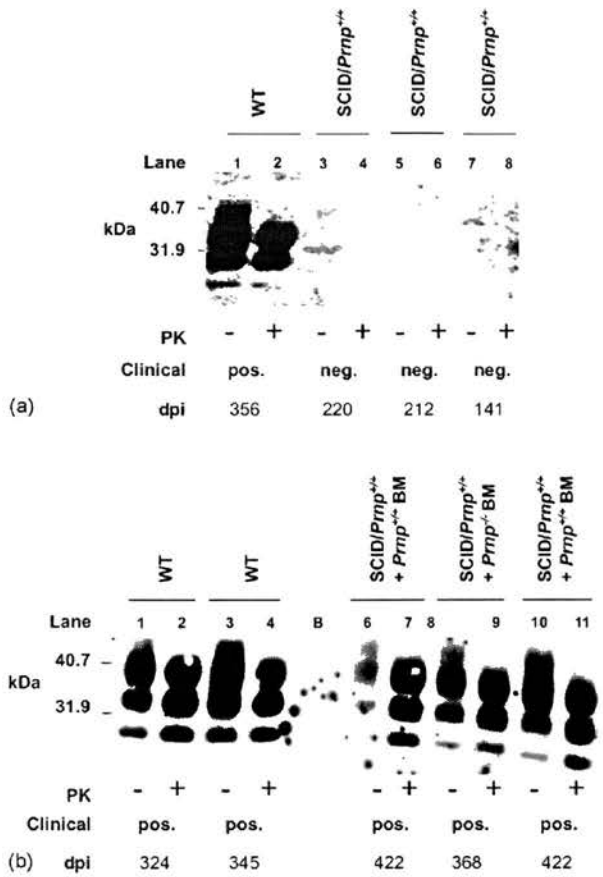


Fig. 5 Immunoblot analysis of spleen tissue from terminally scrapie-affected wild-type mice (WT), SCID/$Prnp^{+/+}$ mice, SCID/$Prnp^{+/+}$ mice reconstituted with $Prnp^{+/+}$ bone marrow (SCID/$Prnp^{+/+}$ + $Prnp^{+/+}$ BM), and SCID/$Prnp^{+/+}$ mice reconstituted with $Prnp^{-/-}$ bone marrow (SCID/$Prnp^{+/+}$ + $Prnp^{-/-}$ BM). Treatment of tissue in the absence (−) or presence (+) of proteinase K (PK) prior to electrophoresis is indicated. After PK treatment, a typical three-band pattern was observed between molecular mass values of 20 and 30 kDa, representing unglycosylated, monoglycosylated, and diglycosylated isomers of PrP (in order of increasing molecular mass). PrP was detected using the PrP-specific monoclonal antiserum 8H4. (a) High levels of PrP^{Sc} were detected in the spleens of terminally scrapie-affected WT mice, but none was detected in tissues from SCID/$Prnp^{+/+}$ mice at any time point. (b) However, high levels of PrP^{Sc} were detected in spleens of SCID/$Prnp^{+/+}$ mice grafted with either $Prnp^{+/+}$ (SCID/$Prnp^{+/+}$ + $Prnp^{+/+}$ BM) or $Prnp^{-/-}$ BM (SCID/$Prnp^{+/+}$ + $Prnp^{-/-}$ BM). Lane B is blank; pos.: mice that developed clinical signs of scrapie; neg.: mice that were free of the signs of scrapie; dpi: day post-inoculation on which the tissues were analysed.

4. Discussion

Previous studies have shown that skin scarification is an effective means of scrapie transmission in immunocompetent mice. However, immunodeficient SCID mice are refractory to scrapie when inoculated by this route, illustrating that a functional immune system is critical for the transmission of scrapie to the CNS following inoculation via the skin [19]. In this study, we have demonstrated that reconstitution of SCID mice with immunocompetent BM restores scrapie replication within lymphoid tissues following skin scarification. This effect coincided with the induction of FDC network maturation within the spleens of grafted SCID mice and subsequent ability to accumulate high levels of scrapie infectivity and PrP^{Sc}. Furthermore, we have shown that following inoculation via the skin, scrapie accumulation in lymphoid tissues and subsequent translocation to the CNS is dependent on mature FDCs but independent of the PrP status of lymphoytes and other BM-derived cells. Taken together, these findings are consistent with previous studies, which demonstrate that following intra-peritoneal inoculation with the ME7 scrapie strain, a functional immune system and more critically PrP-expressing FDCs, are required for transport of the agent from the periphery to the CNS [7,8,13].

SCID mice suffer from a congenital syndrome, which is characterised by the loss of both B- and T-lymphocyte immunity [33]. Secondary to this defect, they also lack functional FDCs as stimulation from lymphocytes is required for the maturation and maintenance of FDCs [34]. Unfortunately, despite careful husbandry due to their dysfunctional immune system, all scrapie-challenged SCID/$Prnp^{+/+}$ mice in this study succumbed to non-TSE related disease up to 274 days post-inoculation. These diseases were non-infectious (e.g. thymic tumours) and were not a reflection of the microbiological status of the husbandry conditions, which were maintained to a high standard of hygiene. Previous data from this laboratory [19] have shown that in contrast to wild-type mice, SCID mice did not succumb to clinical scrapie following exposure to a similar dose of scrapie via skin scarification (mean survival period = 442 ± 21 days post-inoculation, $n = 23$, range = 259–586 days). Following intraperitoneal inoculation with ME7, scrapie PrP^{Sc} is detected in the brain considerably before the onset of clinical signs [29]. In the current study, we measured the levels of disease-specific PrP accumulations in the spleens and brains of all scrapie-inoculated SCID mice. No disease-specific PrP accumulation was detected within the brains or spleens of any of the scrapie-inoculated SCID mice, supporting the assumption that they would not have subsequently developed clinical disease consistent with data from previous studies using this TSE strain [19]. Scrapie infectivity was also undetectable in the spleens of scrapie-inoculated SCID mice,

assayed 220 days post-inoculation. These data are consistent with the hypothesis that following inoculation by skin scarification, scrapie infectivity is unlikely to reach the CNS by direct transport via nerves within the skin or via the bloodstream.

Engraftment of SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM-restored functional lymphocyte populations within the spleen. Furthermore, these lymphocytes were functional as they produced immunoglobulins and were able to stimulate FDC maturation and network formation [33,34]. The development of germinal centre architecture, comparable to immunocompetent animals, coincided with restored scrapie susceptibility and the accumulation of infectivity and PrP^{Sc} in lymphoid tissues of these mice. Thus, these data demonstrate that following inoculation through scarified skin, scrapie accumulates in lymphoid tissues prior to neuroinvasion, as observed with other peripheral routes of exposure [7,9,35,36]. Our studies also demonstrate that PrP^{c} expression on FDCs alone in lymphoid tissues is sufficient to establish scrapie infection. In the presence of PrP^{c}-expressing FDCs, the PrP^{c} status of bone marrow-derived cells had no significant effect on the accumulation of infectivity and PrP^{Sc} in the spleen or on disease incubation period. Further experiments are necessary to determine whether following inoculation via the skin, PrP^{c}-expressing lymphocytes are permissive to scrapie replication in the absence of PrP^{c} expression by FDCs. However, these results are consistent with the demonstration that PrP^{c} expression on FDCs, not lymphocytes, is critical for the peripheral accumulation and transport of scrapie [7,12,37]. Thus, we consider that lymphocytes would likewise be unlikely to play a key role following inoculation via the skin. Although not directly involved in the replication of ME7 scrapie strain, lymphocytes play an important indirect role in pathogenesis by maintaining the maturation of FDC networks within lymphoid tissues [33,34].

Interestingly, grafted SCID/*Prnp*$^{+/+}$ mice did display significantly longer incubation periods in comparison to immunocompetent wild-type mice. Similar results have also been observed in grafted SCID mice inoculated with scrapie strain C506M3 by intra-peritoneal injection [38]. The reason for the delay in the onset of the neurological disease in BM-grafted SCID/*Prnp*$^{+/+}$ mice is not known, but it might be that at the time of scrapie inoculation the restoration of geminal centre functionality in these mice was incomplete. Incomplete reconstitution of SCID/*Prnp*$^{+/+}$ mice might be a consequence of the high natural killer cell activity within SCID mice, which may impair the development of donor BM cells [39]. This effect could have reduced the number of potential peripheral target cells, such as FDCs, available for agent replication at the time of inoculation. In the temporary absence of FDCs at the time of inoculation, both scrapie replication in the spleen and subsequent neuroinvasion are significantly delayed [13]. To achieve efficient reconstitution, it has been suggested that mice are sublethally γ-irradiated prior to cell transfer, to encourage full maturation of grafted bone marrow cells [40]. This procedure was not undertaken in this study as SCID mice have an increased sensitivity to irradiation due a general defect in DNA repair mechanisms, which is believed to be closely linked to the *scid* mutation [41]. It was our concern that γ-irradiation may have adverse affects on the architecture of the skin, blood-brain barrier, or nerve-brain barrier of SCID mice; any of which may have facilitated neuroinvasion by an atypical mechanism. Although neuroinvasion was delayed in the reconstituted SCID/*Prnp*$^{+/+}$ mice, no significant difference was observed in the severity or distribution of vacuolation or disease-specific PrP accumulation within the brains of wild-type and grafted SCID mice, suggesting that neuroinvasion had occurred in these mice via a common pathway.

Although the grafting of SCID/*Prnp*$^{+/+}$ mice with immunocompetent BM-restored scrapie susceptibility in most cases, 5/11 SCID/*Prnp*$^{+/+}$ + *Prnp*$^{+/+}$ BM mice and 2/10 SCID/*Prnp*$^{+/+}$ + *Prnp*$^{-/-}$ BM mice remained free from the signs of scrapie 521 days post-inoculation, at which point the experiment was terminated. ELISA and PCR genotyping analysis suggested that reconstitution had been successful in these surviving mice (data not shown), yet they were refractory to peripheral inoculation. Studies suggest that following BM grafting of SCID mice, it takes approximately 4–6 weeks for full restoration of BM-derived cell populations and germinal centre architecture [40]. As this time period may vary between individual animals, it is conceivable that in this study, the surviving grafted SCID/*Prnp*$^{+/+}$ mice may not have achieved a mature, fully functional immune status, prior to inoculation. This may have prevented scrapie replication establishing due to a lack of functional peripheral target cells such as FDCs [13]. In the absence of mature FDCs at the time of inoculation, it is likely that a significant amount of the inoculum is destroyed by macrophages [42,43]. This delay is again consistent with the hypothesis that scrapie infectivity is unlikely to reach the CNS from the skin by the direct capture of infectivity by nerves within the skin or by direct transport via the bloodstream.

How scrapie is transported from the skin to FDCs within draining lymphoid tissues is not known. Migratory BM-derived Langerhans cells are a plau-

sible candidate mechanism as they acquire antigens in the skin and transport them to lymphoid tissues. Data presented here demonstrate that following inoculation via the skin, the accumulation of scrapie in the spleen and disease incubation period are not affected by the PrP^c status of bone marrow-derived cells. Thus, if scrapie is transported from the skin to lymphoid tissues in a cell-dependent manner, these data suggest that PrP^cexpression by such cells is not critical.

Data presented here demonstrate that scrapie replication in the spleen following inoculation by skin scarification occurs only in the presence of mature FDCs and is independent of the PrP status of surrounding splenic lymphocytes and other bone marrow-derived cells. Furthermore, our results indicate that mature, functional FDCs are required for subsequent neuroinvasion. These data are consistent with previous research using the ME7 scrapie strain, which suggests that FDCs are critical for efficient scrapie neuroinvasion following intra-peritoneal exposure [7,8,13]. Once TSEs spread to the CNS, the neurodegeneration they cause is considered irreversible. The identification of an important role for FDCs in the pathogenesis of disease following skin scarification provides an opportunity for therapeutic intervention prior to neuroinvasion as are already being investigated following other peripheral routes of exposure [13,14,44,45].

Acknowledgements

We thank Jenny Beaton, Lorraine Gray, Irene McConnell and Mary Brady (Institute for Animal Health, Neuropathogenesis Unit, Edinburgh, UK) for excellent technical support; Man-Sun Sy (Case Western Reserve University School of Medicine, Cleveland, OH, USA) for provision of 8H4 monoclonal antiserum. This work was supported by funding from the Medical Research Council and the Biotechnology and Biological Sciences Research Council.

References

[1] Farquhar CF, Somerville RA, Bruce ME. Straining the prion hypothesis. Nature 1998;391:345–6.

[2] Meyer RK, McKinley MP, Bowman KA, Braunfeld MB, Barry RA, Prusiner SB. Separation and properties of cellular and scrapie prion proteins. Proc Natl Acad Sci USA 1986;83:2310–4.

[3] Prusiner SB. Novel proteinacious infectious particles cause scrapie. Science 1982;216:136–44.

[4] Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, et al. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature 1997;389:498–501.

[5] Kitamoto T, Muramoto T, Mohri S, Doh-Ura K, Tateishi J. Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. J Virol 1991;65:6292–5.

[6] McBride P, Eikelenboom P, Kraal G, Fraser H, Bruce ME. PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. J Pathol 1992;168:413–8.

[7] Brown KL, Stewart K, Ritchie D, Mabbott NA, Williams A, Fraser H, et al. Scrapie replication in lymphoid tissues depends on PrP-expressing follicular dendritic cells. Nat Med 1999;5:1308–12.

[8] Mabbott NA, Williams A, Farquhar CF, Pasparakis M, Kollias G, Bruce ME. Tumour necrosis factor-alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie. J Virol 2000;74:3338–44.

[9] van Keulen LJM, Schreuder BEC, Meloen RH, Mooij-Harkes G, Vromans MEW, Langeveld JPM. Immunohistological detection of prion protein in lymphoid tissues of sheep with natural scrapie. J Clin Microbiol 1996;34:1228–31.

[10] Hill AF, Butterworth RJ, Joiner S, Jackson G, Rossor MN, Thomas DJ, et al. Investigation of variant Creutzfeldt-Jakob disease and other prion diseases with tonsil biopsy samples. Lancet 1999;353:183–9.

[11] Fraser H, Brown KL, Stewart K, McConnell I, McBride P, Williams A. Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone marrow. J Gen Virol 1996;77:1935–40.

[12] Klein MA, Frigg R, Raeber AJ, Flechsig E, Hegyi I, Zinkernagel RM, et al. PrP expression in B lymphocytes is not required for prion neuroinvasion. Nat Med 1998; 4:1429–33.

[13] Mabbott NA, Mackay F, Minns F, Bruce ME. Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. Nat Med 2000;6:719–20.

[14] Montrasio F, Frigg R, Glatzel M, Klein MA, Mackay F, Aguzzi A, et al. Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. Science 2000;288:1257–9.

[15] Glatzel M, Heppner FL, Albers KM, Aguzzi A. Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion. Neuron 2001;31:25–34.

[16] Duffy P, Wolf J, Collins G, DeVoe AG, Streffin B, Cowen D. Possible person-to-person transmission of Creutzfeldt-Jacob disease. N Engl J Med 1974;290:692.

[17] Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. Lancet 2000;356:999.

[18] Hunter N, Foster J, Chong A, McCutcheon S, Parnham D, Eaton S, et al. Transmission of prion diseases by blood transfusion. J Gen Virol 2002;83:2897–905.

[19] Taylor DM, McConnell I, Fraser H. Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. J Gen Virol 1996;77:1595–9.

[20] Brotherston JG, Renwick CC, Stamp JT, Zlotnik I. Spread of scrapie by contact to goats and sheep. J Comp Pathol 1968;78:9–17.

[21] Bartz JC, Kincaid AE, Bessen RA. Rapid prion neuroinvasion following tongue infection. J Virol 2003;77:583–91.

[22] Flechsig E, Hegyi I, Enari M, Scwarz P, Collinge J, Weissmann C. Transmission of scrapie by steel-surface-bound prions. Mol Med 2001;7:679–84.

[23] Birmingham K. Were some CJD victims infected by vaccines? Nature 2000;408:3–5.

[24] Lupi O. Prions in dermatology. J Am Acad Dermatol 2002;46:790–3.

[25] Büeler H, Aguzzi A, Sailer A, Greiner R-A, Autenried P, Aguet M, et al. Mice devoid of PrP are resistant to scrapie. Cell 1993;73:1339–47.
[26] Manson JC, Clarke AR, Hooper ML, Aitchison L, McConnell I, Hope J. 129/*Ola* mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. Mol Neurobiol 1994;8:121–7.
[27] Fraser H, Dickinson AG. Agent-strain differences in the distribution and intensity of grey matter vacuolation. J Comp Pathol 1973;83:29–40.
[28] Dickinson AG, Meikle VM, Fraser H. Genetical control of the concentration of ME7 scrapie agent in the brain of mice. J Comp Pathol 1969;79:15–22.
[29] Farquhar CF, Dornan J, Somerville RA, Tunstall AM, Hope J. Effect of Sinc genotype, agent isolate and route of infection on the accumulation of protease-resistant PrP in non-central nervous system tissues during the development of murine scrapie. J Gen Virol 1994;75:495–504.
[30] Zanusso G, Liu D, Ferrari S, Hegyi I, Yin X, Aguzzi A, et al. Prion protein expression in different species: analysis with a panel of new mAbs. Proc Natl Acad Sci USA 1998;95: 8812–6.
[31] Kapasi ZF, Qin D, Kerr WG, Kosco-Vilbois MH, Schultz LD, Tew JG, et al. Follicular dendritic cell (FDC) precursors in primary lymphoid tissues. J Immunol 1998;160:1078–84.
[32] Tkachuk M, Bolliger S, Ryffel B, Pluschke G, Banks TA, Herren S, et al. Crucial role of tumour necrosis factor receptor 1 expression on nonhematopoietic cells for B cell localization within the splenic white pulp. J Exp Med 1998;187:469–77.
[33] Bosma GC, Custer RP, Bosma ML. A severe combined immunodeficiency mutation in the mouse. Nature 1983;301:1339–47.
[34] Kapasi ZF, Burton GF, Schultz LD, Tew JG, Szakal AK. Induction of functional follicular dendritic cell development in severe combined immunodeficiency mice. J Immunol 1993;150:2648–58.
[35] Hilton D, Fathers E, Edwards P, Ironside J, Zajicek J. Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease. Lancet 1998;352:703–4.
[36] Sigurdson CJ, Williams ES, Miller MW, Spraker TR, O'Rourke KI, Hoover EA. Oral transmission and early lymphoid tropism of chronic wasting disease PrP^{res} in mule deer fawns (*Odocoileus hemionus*). J Gen Virol 1999;80:2757–64.
[37] Montrasio F, Cozzio A, Flechsig E, Rossi D, Klein MA, Rulicke T, et al. B-lymphocyte-restricted expression of the prion protein does not enable prion replication in PrP knockout mice. Proc Natl Acad Sci USA 2001;98:4034–7.
[38] Lasmézas CI, Cesbron J-Y, Deslys J-P, Demaimay R, Adjou KT, Rioux R, et al. Immune system-dependent and independent replication of the scrapie agent. J Virol 1996;70:1292–5.
[39] Philips RA, Fulop GM. Pleiotropic effects of the scid mutation: effects on lymphoid differentiation and or repair of radiation damage. In: Bosma MJ, Philips RA, Schuler G, editors. The scid mouse: characterisation and potential uses. Springer-Verlag; 1989. p. 11–17.
[40] Fulop GM, Philips RA. Full reconstitution of the immune deficiency in SCID mice with normal stem cells requires low-dose irradiation of the recipients. J Immunol 1986;136:4438–43.
[41] Fulop GM, Philips RA. The scid mutation in mice causes a general defect in DNA repair. Nature 1990;347:479–82.
[42] Carp RI, Callahan SM. Effect of mouse peritoneal macrophages on scrapie infectivity during extended in vitro incubation. Intervirology 1982;17:201–7.
[43] Beringue V, Demoy M, Lasmezas CI, Gouritin B, Weingarten C, Deslys J-P, et al. Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis. J Pathol 2000;190:495–502.
[44] Mabbott NA, Bruce ME, Botto M, Walport MJ, Pepys MB. Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie. Nat Med 2001;7:485–7.
[45] Mabbott NA, McGovern G, Jeffrey M, Bruce ME. Temporary blockade of the tumour necrosis factor signaling pathway impedes the spread of scrapie to the brain. J Virol 2002;76:5131–9.

IMM 2074 B
Journal Name Manuscript No.
Dispatch: 22.11.04 Journal: IMM CE: Blackwell
Author Received: No. of pages: 10 PE: Dipu
IMMUNOLOGY ORIGINAL ARTICLE

Follicular dendritic cell dedifferentiation reduces scrapie susceptibility following inoculation via the skin

Joanne Mohan, Moira E. Bruce & Neil A. Mabbott
Institute for Animal Health, Ogston Building, Edinburgh, United Kingdom

Summary

Transmissible spongiform encephalopathies (TSEs) are a group of subacute infectious neurodegenerative diseases that are characterized by the accumulation in affected tissues of PrP^{Sc}, an abnormal isoform of the host prion protein (PrP^{c}). Following peripheral exposure, TSE infectivity and PrP^{Sc} usually accumulate in lymphoid tissues prior to neuroinvasion. Studies in mice have shown that exposure through scarified skin is an effective means of TSE transmission. Following inoculation via the skin, a functional immune system is critical for the transmission of TSEs to the brain, but until now, it has not been known which components of the immune system are required for efficient neuroinvasion. Temporary dedifferentiation of follicular dendritic cells (FDCs) by treatment with an inhibitor of the lymphotoxin-β receptor signalling pathway (LTβR-Ig) 3 days before or 14 days after inoculation via the skin, blocked the early accumulation of PrP^{Sc} and TSE infectivity within the draining lymph node. Furthermore, in the temporary absence of FDCs before inoculation, disease susceptibility was reduced and survival time significantly extended. Treatment with LTβR-Ig 14 days after TSE inoculation also significantly extended the disease incubation period. However, treatment 42 days after inoculation did not affect disease susceptibility or survival time, suggesting that the infection may have already have spread to the nervous system. Together these data show that FDCs are essential for the accumulation of PrP^{Sc} and infectivity within lymphoid tissues and subsequent neuroinvasion following TSE exposure via the skin.

Keywords: follicular dendritic cell; lymphotoxin; scrapie; skin, transmissible spongiform encephalopathy

doi:10.1111/j.1365-2567.2004.02074.x
1 Received 13 October 2004; revised ?????? Month 2004; accepted 15 October 2004.
Correspondence: Dr N. A. Mabbott, Institute for Animal Health, West Mains Road, Edinburgh EH9 3JF, United Kingdom.
E-mail: neil.mabbott@bbsrc.ac.uk
8 Senior author: ??

Introduction

The transmissible spongiform encephalopathies (TSEs), or prion diseases, are subacute neurodegenerative diseases that affect humans and both wild and domestic animals. Most TSEs, including natural sheep scrapie, bovine spongiform encephalopathy (BSE), chronic wasting disease in mule deer and elk, and kuru and variant Creutzfeldt–Jakob disease (vCJD) in humans, are acquired by peripheral exposure. Although the main route of transmission of BSE to cattle and other species is considered to be oral (ingestion), other routes of TSE transmission have been identified. Accidental iatrogenic transmissions of sporadic (s)CJD to patients have occurred through the transplantation of sCJD-contaminated tissues or via pituitary-derived hormones.[1] Recent evidence also indicates that vCJD in humans has been transmitted via blood transfusion.[2,3] Studies in mice have shown that skin scarification is an effective means of scrapie transmission, highlighting another potential route of accidental transmission.[4] Therefore, it is possible that some cases of natural sheep scrapie might be transmitted through skin lesions either in the mouth[5] or during close contact,[6] or be passed from mother to offspring through sites of skin trauma at birth. Surgical instruments contaminated with sCJD infectivity have also been shown to have the potential to transmit disease.[7] Together, these examples highlight important health and safety issues concerning risks to patients, health workers and scientists of acquiring disease. Biopharmaceutical and cosmetic products derived from sheep and cattle tissues might also have the potential to transmit disease when applied to skin lesions.[8,9]

Understanding the immunobiology of scrapie transmission via the skin will aid the determination of risk and the development of therapeutic strategies.

The host prion protein (PrP^c) is critical for TSE agent replication and accumulates as an abnormal, detergent-insoluble, relatively proteinase-resistant isoform, PrP^{Sc}, in diseased tissues.[10] PrP^{Sc}, or an intermediate between PrP^c and PrP^{Sc}, is considered to constitute a major, or possibly the sole, component of the infectious agent.[11] Once TSEs infect the central nervous system (CNS), the accumulation of PrP^{Sc} is accompanied by neurodegeneration and, ultimately, the death of the host. Following peripheral inoculation, TSE agents usually accumulate in lymphoid tissues prior to the dissemination of infection to the CNS. Within the lymphoid tissues of TSE-infected hosts,[12–16] PrP^{Sc} accumulation initially takes place in germinal centres in association with follicular dendritic cells (FDCs). Studies in rodents inoculated intraperitoneally with scrapie have shown that mature FDCs are critical for scrapie accumulation in lymphoid tissues, and in their absence neuroinvasion is significantly impaired.[12,13,17] From the lymphoid tissues, infectivity spreads to the CNS via peripheral nerves.[18,19]

Previous studies have shown that a functional immune system is critical for scrapie neuroinvasion following inoculation by skin scarification, as mice with severe combined immunodeficiency (SCID) do not accumulate PrP^{Sc} and infectivity in their spleens, or develop clinical disease when inoculated with scrapie by this route.[4,20] SCID mice are indirectly deficient in FDCs as they require important stimuli from lymphocytes for their maturation.[21] The induction of FDC development in SCID mice by bone marrow grafting restores the accumulation of scrapie in the spleen after inoculation via the skin.[20] However, whether FDCs or other components of the immune system are required for scrapie neuroinvasion following inoculation via the skin is not known. In an experimental system, migratory bone marrow-derived dendritic cells have been shown to have the potential to deliver scrapie infectivity directly to the nervous system.[22] As skin is highly innervated, we considered that neuroinvasion might occur via an FDC-independent pathway. For example, lymphocytes or Langerhans' cells might acquire scrapie within the skin and transport it directly to local peripheral nerves. Therefore, in this study experiments were performed to investigate whether FDCs are required for scrapie neuroinvasion after inoculation via the skin.

Materials and methods

Treatment with lymphotoxin β-receptor (LTβR) immunoglobulin

C57BL/Dk mice (8–12 weeks old) were given a single intraperitoneal (i.p.) injection of a fusion protein containing the soluble LTβR domain linked to the Fc portion of human immunoglobulin G1 (IgG1) (LTβR-Ig)[23] or with 100 μg of polyclonal human IgG (hu-Ig) (Sandoglobulin®) as a control.

Scrapie inoculation

Mice were inoculated with the ME7 strain of scrapie by skin scarification of the medial surface of the right thigh. Briefly, prior to scarification ≈ 1 cm^2 of hair covering the scarification site was trimmed using curved scissors and then removed completely with an electric razor. Twenty-four hours later, a 23-gauge needle was used to create a 5-mm long abrasion in the epidermal layers of the skin at the scarification site. Care was taken to avoid damage to the dermis or to draw blood during scarification. Then, using a 26-gauge needle, one droplet (≈ 6 μl) of ME7 scrapie inoculum, from a 1% or 0·1% (w/v) terminal scrapie mouse brain homogenate in physiological saline, was applied to the abrasion and worked into the site using sweeping strokes. The scarification site was then sealed with OpSite (Smith & Nephew Medical Ltd, Hull, UK) and allowed to dry before the animals were returned to their final holding cages. Following challenge, the animals were coded, assessed weekly for signs of clinical disease and killed at a standard clinical end-point.[24] Scrapie diagnosis was confirmed by histopathological assessment of TSE vacuolation in the brain.

At the time-points indicated, some mice were killed and their spleens and inguinal lymph nodes (ILNs) were taken for further analysis. For bioassay of scrapie infectivity, two half spleens were pooled from each treatment group and prepared as 10% (w/v) homogenates in physiological saline. Likewise, the ILNs draining the inoculation site were pooled from two mice and prepared as a 10% (w/v) homogenate. Groups of 12 C57BL/Dk indicator mice were injected intracerebrally (i.c.) with 20 μl of each homogenate. The scrapie titre in each sample was determined from the mean incubation period in the assay mice, by reference to established dose/incubation period response curves for ME7 scrapie-infected spleen tissue, as previously described.[25]

Immunohistochemical analysis

To monitor the effects of treatment on FDC status, ILNs and half spleens were taken from two mice in each group and snap-frozen at the temperature of liquid nitrogen. Serial frozen sections (thickness, 10 μm) were cut on a cryostat and FDCs were visualized by staining with either the FDC-specific rat monoclonal antiserum, FDC-M2 (AMS Biotechnology, Oxon, UK), or 8C12 monoclonal antiserum to detect CD35 (BD Biosciences PharMingen, Oxford, UK). Immunolabelling was carried out by using alkaline phosphatase coupled to the avidin–biotin

complex (Vector Laboratories, Burlingame, CA, USA). Vector Red (Vector Laboratories) was used as a substrate.

For the detection of PrP in the brain, tissues were fixed in periodate–lysine–paraformaldehyde and embedded in paraffin wax. Sections (6-μm thickness) were deparaffinized and pretreated to enhance PrP immunostaining by hydrated autoclaving (15 min, 121°, hydration) and subsequent immersion in formic acid (98%) for 5 min.[26] Sections were then stained with the PrP-specific monoclonal antiserum, 6H4 (Prionics, Zürich, Switzerland), and immunolabelling was detected by using hydrogen peroxidase coupled to the avidin–biotin complex (Vector Laboratories), with diaminobenzidine (DAB) as a substrate. Glial fibrillary acid protein (GFAP) was detected on adjacent brain sections by using rabbit GFAP-specific antiserum (DAKO Ltd, Ely, UK), and immunolabelling was carried out by using alkaline phosphatase coupled to the avidin–biotin complex with Vector Red as a substrate.

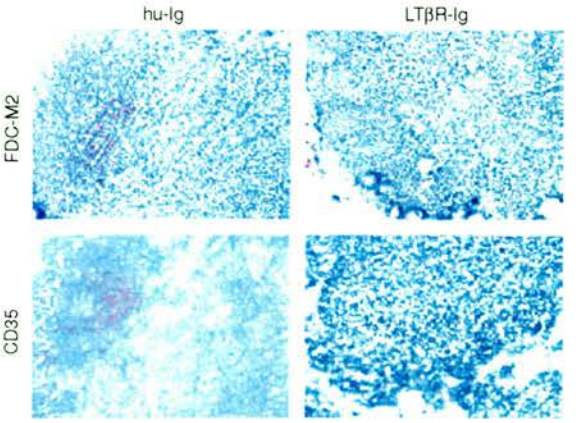
All sections were counterstained with haematoxylin to distinguish cell nuclei.

Paraffin-embedded tissue (PET) immunoblot detection of PrP^{Sc}

PrP^{Sc} was detected in PET sections of spleen and ILNs, as previously described.[27] Briefly, tissues were fixed in periodate–lysine–paraformaldehyde and embedded in paraffin wax. Serial sections (6-μm thickness) were mounted on poly(vinylidine difluoride) membrane (Bio-Rad, Hemel Hempstead, UK) and fixed by incubation at 55° overnight. Membranes were then deparaffinized and digested with proteinase K (20 μg/ml) for 16 hr at 55° (to confirm the presence of PrP^{Sc}), washed in TBS/Tween [10 mM Tris–HCl pH 7·8, 100 mM NaCl, 0·5% (v/v) Tween] and denatured in 3 M guanidine isothiocyanate (10 mM Tris–HCl, pH 7·8) for 10 min. Membranes were blocked in 2% casein, and PrP was detected with the PrP-specific rabbit polyclonal antiserum, 1B3,[28] followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Bound alkaline phosphatase activity was detected by using SigmaFast™ nitroblue tetrazolium/5-bromo-4-chloroindol-2-yl phosphate (NBT/BCIP) solution (Sigma, Poole, UK). Immunostained membranes were assessed using an Olympus dissecting microscope.

Statistical analysis

Where indicated, incubation periods are presented as mean (days) ± standard error (SE). Significant differences between incubation periods in different groups were determined by using one-way analysis of variance (ANOVA).



COLOUR FIG.

Figure 1. Effect of treatment with lymphotoxin β-receptor immunoglobulin (LTβR-Ig) on follicular dendritic cell (FDC) status in the inguinal lymph nodes of uninfected mice. Tissues were taken 3 days after injection with polyclonal human immunoglobulin G (hu-Ig) (control) or LTβR-Ig, and adjacent frozen sections were stained with the FDC-specific monoclonal antiserum FDC-M2 (top row; red) and 8C12 antiserum to detect CD35 (bottom row; red). Expression of FDC-M2 and CD35 were undetectable in inguinal lymph nodes after treatment with LTβR-Ig. All sections were counterstained with haematoxylin (blue). Original magnification × 200.

Results

Effect of LTβR-Ig treatment on FDC status in ILNs

The maintenance of FDCs in a differentiated state requires continual stimulation through the LTβR, as mature cells rapidly dedifferentiate when this signalling pathway is blocked.[29] Here, temporary blockade of the LTβR signalling pathway was achieved by a single i.p. injection of 100 μg of LTβR-Ig.[23] Within 3 days of treatment with LTβR-Ig, expression of the FDC markers FDC-M2 and CD35 (complement receptor 1) was undetectable in ILNs (Fig. 1) and in spleen (data not shown). The effects of treatment with LTβR-Ig on FDC status are temporary, lasting $\approx$28 days.[29,30] Treatment with 100 μg of hu-Ig, as a control, had no adverse effect on FDC status in ILNs (Fig. 1) or in the spleen (data not shown).

Effect of LTβR-Ig treatment on the early accumulation of PrP^{Sc} within ILNs and the spleen

Following peripheral inoculation of mice with the ME7 strain of scrapie, high levels of PrP^{Sc} and infectivity accumulate in lymphoid tissues within the first few weeks post-inoculation, and these levels are maintained throughout the course of infection.[12,13,31] In the present study, mice were treated with LTβR-Ig or hu-Ig (as a control), 14 or 42 days after inoculation with scrapie by skin scarification, and spleens and both ILNs were taken from two mice of each treatment group 3 days later (days 17 and 45, respectively).

PrP^{Sc} accumulations within these tissues were detected by PET immunoblot analysis. PrP^{Sc} was detected within two or three lymphoid follicles of the draining (right) ILNs of hu-Ig-treated control mice, 17 days after inoculation with scrapie (Table 1, treatment on day 14). The cellular distribution of the PrP^{Sc} was consistent with its accumulation on FDCs.[13] No PrP^{Sc} was detected in the non-draining (left) ILNs or spleen 17 days after inoculation (Table 1, treatment on day 14). By 45 days after inoculation with scrapie, PrP^{Sc} was present in a greater number of lymphoid follicles in the draining ILNs of control-treated mice (Table 1, treatment on day 42; Fig. 2a). Furthermore, PrP^{Sc} was also detectable in a single lymphoid follicle in the left ILN (Table 1) and in spleen (Table 1, treatment on day 42; Fig. 2c) at this time-point. These data demonstrate that following inoculation via the skin, PrP^{Sc} accumulates first upon FDCs in the draining ILN and subsequently spreads to other non-draining lymph nodes and spleen between 17 and 42 days after inoculation.

When mice were treated with LTβR-Ig, the number of follicles containing PrP^{Sc} in the draining ILN and spleen were visibly reduced or completely absent 3 days after treatment (Table 1; Fig. 2b, 2d, respectively). Therefore, the temporary dedifferentiation of FDCs correlated with a rapid reduction in the number of PrP^{Sc}-positive lymphoid follicles in ILNs and the spleen.

Table 1. The effect of treatment with lymphotoxin β-receptor immunoglobulin (LTβR-Ig) on the early accumulation of PrP^{Sc} in the inguinal lymph nodes and spleens of scrapie-inoculated mice*†

Day of	hu-Ig			LTβR-Ig		
treatment	Right ILN‡	Left ILN§	Spleen	Right ILN	Left ILN	Spleen
14	++	–	–	+/–	–	–
42	+++	+	+	+/–	–	+/–

*Mice were given a single intraperitoneal (i.p.) injection (100 μg) of LTβR-Ig or polyclonal human immunoglobulin G (hu-Ig), as a control, on the days indicated after inoculation with scrapie via skin scarification of the right thigh.

†Tissues were taken 3 days after treatment, and the number of PrP^{Sc}-containing lymphoid follicles in inguinal lymph nodes (ILNs) and spleen from two mice was scored as follows: +++, ≥ 4 positive follicles; ++, 2–3 positive follicles; +, 1 positive follicle; +/–, 1 positive follicle in only one of the samples; –, no PrP^{Sc} detected.

‡Inguinal lymph node draining the site of inoculation.

§Non-draining inguinal lymph node.

COLOUR FIG.

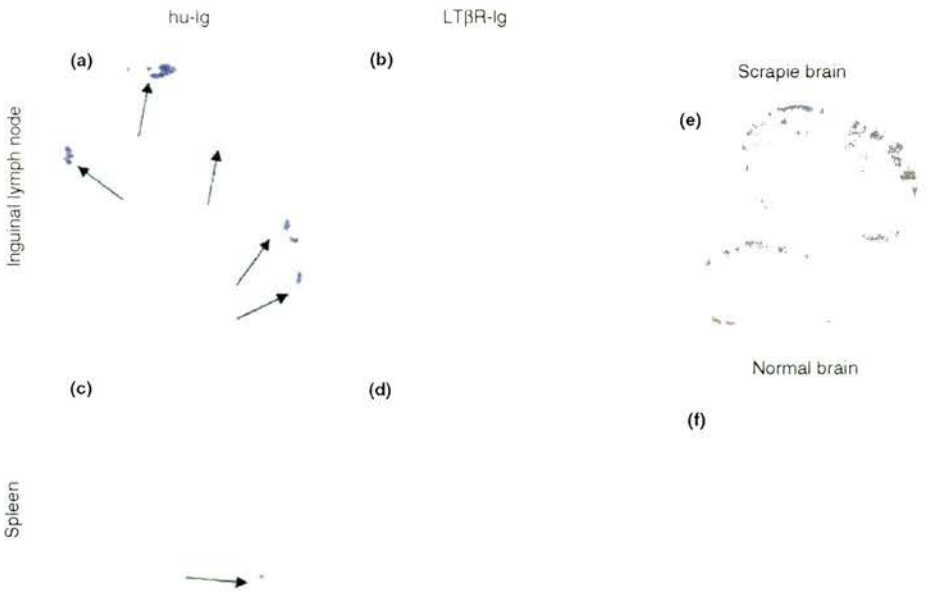


Figure 2. Effect of treatment with lymphotoxin β-receptor immunoglobulin (LTβR-Ig) on the early accumulation of PrP^{Sc} in the draining inguinal lymph node and in the spleen. Mice were given a single intraperitoneal (i.p.) injection of LTβR-Ig or polyclonal human immunoglobulin G (hu-Ig) (control) 42 days after inoculation with scrapie via skin scarification on the right thigh. Tissues from two mice from each group were collected 3 days after treatment, and PrP^{Sc} accumulations were determined by paraffin-embedded tissue (PET) immunoblotting. Abundant PrP^{Sc} accumulations were detected in the lymphoid follicles of polyclonal human immunoglobulin G (hu-Ig)-treated animals (a and c, dark staining, arrowheads). In contrast, PrP^{Sc} accumulations were undetectable in tissues from LTβR-Ig-treated mice (b and d). Terminally scrapie affected brain tissue (e) and uninfected normal brain tissue (f) were included as controls to confirm the specificity of PrP^{Sc} detection.

Effect of LTβR-Ig treatment on the early accumulation of infectivity within ILNs and the spleen

The draining ILNs and spleens were taken from two mice of each control and LTβR-Ig-treatment group 70 days after scrapie inoculation by skin scarification. The scrapie infectivity titre in pooled ($n = 2$) tissue homogenates was estimated by bioassay in groups of indicator mice. As expected, ILNs from each group of hu-Ig-treated control mice contained high infectivity titres [6·3–6·7 log i.c. 50% infective dose (ID_{50})/g; Table 2]. In the draining ILNs of mice treated with LTβR-Ig 3 days before scrapie inoculation, infectivity was undetectable, suggesting a scrapie infectivity titre, if present, of < 3·5 log i.c. ID_{50}/g (at least 1000-fold less than the level detected in ILNs of control-treated mice assayed at the same time postinoculation; Table 2). Thus, temporary FDC dedifferentiation before inoculation with scrapie via the skin blocks the early accumulation of infectivity in the draining ILN. The mean incubation periods obtained following injection of pooled lysates of ILNs from mice treated with LTβR-Ig 14 or 42 days after inoculation, were significantly longer than those obtained following injection of lysates from control mice ($P < 0·01$ and $P < 0·001$, $n = 9$, ANOVA, respectively; Table 2). Therefore, ILNs from mice treated with LTβR-Ig 14 or 42 days after inoculation, contained detectable but significantly lower levels of infectivity than those measured in ILNs from control-treated mice (Table 2).

Similarly, spleens taken from each group of control-treated mice 70 days after inoculation contained high levels of scrapie infectivity (6·0–7·1 log i.c. ID_{50}/g; Table 3). However, after treatment with LTβR-Ig 3 days before or 14 days after inoculation with scrapie, infectivity was undetectable in the spleen, suggesting a scrapie infectivity titre, if present, of < 3·5 log i.c. ID_{50}/g (at least 1000-fold less than the level detected in spleens of control-treated

Table 2. Effect of treatment with lymphotoxin β-receptor immunoglobulin (LTβR-Ig) on the accumulation of scrapie infectivity in the draining inguinal lymph node 70 days after inoculation with scrapie via the skin†

	hu-Ig			LTβR-Ig		
Day of treatment	Incidence‡	Mean incubation period (days) ± SE	Titre§	Incidence	Mean incubation period (days) ± SE	Titre
−3	7/7	196 ± 5	6·6	0/9	9× > 300	< 3·5
+14	7/7	193 ± 6	6·7	8/9	217 ± 3*	5·5
+42	8/8	203 ± 4	6·3	6/8	249 ± 4**	4·1

†Mice were given a single intraperitoneal (i.p.) injection (100 μg) of LTβR-Ig or polyclonal human immunoglobulin G (hu-Ig) as a control on the days indicated before or after inoculation with scrapie via skin scarification of the right thigh. Inginal lymph nodes draining the site of inoculation were pooled from two mice and infectivity levels were determined by intracerebral (i.c.) injection of lysates into groups of C57BL/Dk indicator mice.

‡Incidence = number of animals affected/number of animals tested. The notation 'n × > 300' means that mice were free of the signs of scrapie up to at least this time-point after inoculation.

§Scrapie infectivity titres expressed as log i.c. 50% infectious dose (ID_{50})/g.

*$P < 0·01$, when compared to the mean incubation period for hu-Ig control tissues.

**$P < 0·001$, when compared to the mean incubation period for hu-Ig control tissues.

Table 3. Effect of treatment with lymphotoxin β-receptor immunoglobulin (LTβR-Ig) on the accumulation of scrapie infectivity in the spleen 70 days after inoculation with scrapie via the skin†

	hu-Ig			LTβR-Ig		
Day of treatment	Incidence‡	Mean incubation period (days) ± SE	Titre§	Incidence	Mean incubation period (days) ± SE	Titre
−3	9/9	187 ± 3	7·1	0/9	9× > 300	< 3·5
+14	8/8	188 ± 4	7·0	0/8	9× > 300	< 3·5
+42	9/9	202 ± 9	6·0	6/9	243 ± 5*, 3× > 300	= 4·1

†Mice were given a single i.p. injection (100 μg) of LTβR-Ig or polyclonal human immunoglobulin G (hu-Ig) as a control on the days indicated after inoculation with scrapie via skin scarification of the right thigh. Spleens were pooled from two mice and infectivity levels were determined by intracerebral (i.c.) injection of lysates into groups of C57BL/Dk indicator mice.

‡See Table 2, footnote‡.

§See Table 2, footnote§.

*$P < 0·02$, when compared to the mean incubation period for hu-Ig control tissues.

mice assayed at the same time postinoculation; Table 3). However, comparisons of mean incubation periods obtained following injection of spleen lysates from mice treated 42 days after inoculation, suggested that spleens from LTβR-Ig-treated mice contained detectable, but significantly lower, levels of infectivity (at least 100-fold less; $P < 0{\cdot}02$, $n = 9$, ANOVA) than those measured in spleens from control-treated mice (Table 3).

PrP^{Sc} accumulation within ILNs and the spleen at the terminal stage of disease

As expected, abundant PrP^{Sc} was detected by immunoblot analysis in ILNs and spleens from all hu-Ig-treated control animals that developed clinical signs of scrapie (data not shown). The effects of LTβR-Ig treatment on FDC status are temporary, and mature networks reappear ≈ 28 days later.[29,30] Therefore, the detection of abundant PrP^{Sc} in ILNs and spleens of LTβR-Ig-treated animals that developed clinical signs of scrapie (data not shown) is consistent with the replication of residual infectivity on recovered FDC networks in these tissues.

Effect of LTβR-Ig treatment on scrapie susceptibility

Mice were given a single i.p. injection of LTβR-Ig or hu-Ig (as a control) at one of the following three timepoints relative to scrapie inoculation by skin scarification: 3 days before scrapie inoculation (so that mature FDCs would be absent in lymphoid tissues at the time of challenge); 14 days after inoculation (i.e. soon after the onset of scrapie accumulation in the draining ILN; Table 1); or 42 days after inoculation, when abundant PrP^{Sc} (Table 1; and Fig. 2a) and infectivity (J. Mohan *et al.* unpublished) are detectable in the draining ILN.

All control mice treated with hu-Ig 3 days before scrapie inoculation succumbed to disease with a mean incubation period of 320 ± 8 days ($n = 6$; Table 4). In contrast, LTβR-Ig-treatment 3 days prior to scrapie inoculation reduced disease susceptibility and significantly extended the survival time (Table 4). Four of seven LTβR-Ig-treated mice remained free of the signs of scrapie for up to at least 480 days postinoculation. However, three of seven LTβR-Ig-treated mice did succumb to scrapie after individual incubation periods of 350, 381 and 402 days. These incubation periods were beyond the range seen in the hu-Ig-treated control mice (309–327 days), and the mean incubation period (378 ± 15 days, $n = 6$) was significantly longer than the mean incubation period for control mice ($P < 0{\cdot}001$, ANOVA). Characteristic spongiform pathology, disease-specific PrP accumulation and reactive astrocytes expressing high levels of GFAP were detected in the brains of all control and LTβR-Ig-treated mice that developed clinical scrapie (Fig. 3). In contrast, spongiform pathology, disease-specific PrP accumulation or reactive astrocytes were not detected in the brains of the surviving LTβR-Ig-treated mice (Fig. 3), indicating that infection had not spread to the nervous system in these surviving mice.

Table 4. Effect of treatment with lymphotoxin β-receptor immunoglobulin (LTβR-Ig) on scrapie susceptibility following inoculation via the skin†

	hu-Ig		LTβR-Ig	
Day of treatment	Incidence‡	Mean incubation period (days) ± SE	Incidence	Mean incubation period (days) ± SE
−3	6/6	320 ± 3	3/7	378 ± 15*, 4× > 480
+14	8/8	316 ± 3	8/8	351 ± 4**
+42	7/7	328 ± 6	8/8	343 ± 8***

†Mice were given a single intraperitoneal (i.p.) injection (100 μg) of LTβR-Ig or human immunoglobulin G (hu-Ig) as a control on the days indicated before or after inoculation with scrapie via skin scarification of the right thigh.

‡See Table 2, footnote‡.

*$P < 0{\cdot}001$, when compared to hu-Ig controls.

**$P < 0{\cdot}000005$, when compared to hu-Ig controls.

***$P = 0{\cdot}158$, when compared to hu-Ig controls.

All mice treated with LTβR-Ig 14 days after inoculation developed clinical disease with a significantly extended incubation period when compared to control-treated mice ($P < 0{\cdot}000005$, $n = 8$, ANOVA; Table 4). However, treatment with LTβR-Ig 42 days after scrapie inoculation had no significant effect on the survival time when compared to control-treated mice ($P = 0{\cdot}158$, $n = 8$, ANOVA; Table 4).

Discussion

In order to determine the involvement of FDCs in scrapie pathogenesis following inoculation via the skin, these cells were temporarily dedifferentiated through the blockade of the LTβR signalling pathway, either before, or shortly after, scrapie inoculation. Data presented here show that treatment with LTβR-Ig blocked the early accumulation of scrapie in the draining ILN and spleen. These effects coincided with the temporary dedifferentiation of FDCs in lymphoid tissues. When given 3 days before scrapie inoculation, LTβR-Ig reduced disease susceptibility and extended the survival time when compared to control-treated mice. No pathological signs of scrapie were detected in the brains of mice surviving treatment with LTβR-Ig, confirming that neuroinvasion does not occur by the direct uptake of infectivity by peripheral nerves in the skin. Thus, FDCs are critical for the transmission of scrapie from the skin to the CNS. Whereas treatment with LTβR-Ig 14 days after scrapie inoculation also significantly extended the urvival

COLOUR FIG.

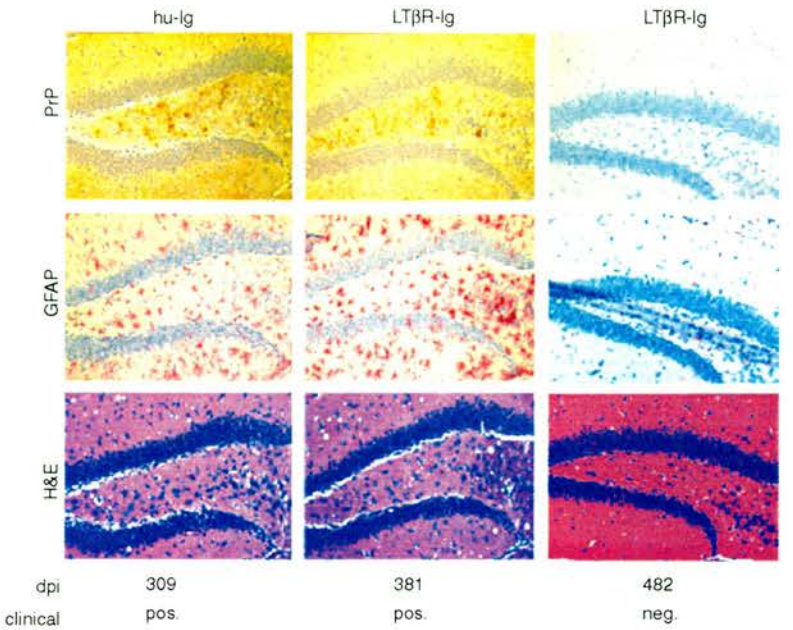


Figure 3. Immunohistological analysis of brain tissue from mice treated with polyclonal human immunoglobulin G (hu-Ig) (control) or with lymphotoxin β-receptor immunoglobulin (LTβR-Ig) 3 days before scrapie inoculation by skin scarification. Large disease-specific PrP accumulations (upper row; brown), and reactive astrocytes expressing high levels of glial fibrillary acid protein (GFAP) (middle row; red) and spongiform pathology (haematoxylin & eosin, lower row) were detected in the hippocampi of all mice showing clinical signs of scrapie. In contrast, in the brains of LTβR-Ig-treated mice that remained free of the clinical signs of disease, no evidence of PrP accumulation, reactive astrocytes or spongiform pathology was detected 480 days after inoculation. All sections were counterstained with haematoxylin (blue). Original magnification $\times 200$. dpi, days postinoculation on which the tissues were analysed; pos., mice that developed clinical signs of scrapie; neg., mice that were free of the clinical signs of scrapie.

time, treatment 42 days after scrapie inoculation did not, suggesting that infectivity had already spread to the peripheral nervous system by this time.

Lymphocytes provide essential cytokine signals for FDC development and maturation,[21,32,33] as immunodeficient mice that lack expression of lymphotoxin (LT)α[34] or LTβ[35] lack mature FDC networks. Lymphocytes express these cytokines as a membrane-bound heterotrimer (LT$\alpha_1\beta_2$), which signals through LTβR on FDCs or their precursors.[36] FDCs likewise do not develop in LTβR-deficient mice.[37] FDC networks require continual LTβR stimulation as they rapidly collapse from their mature state when LTβR signalling is specifically blocked by treatment with LTβR-Ig (Fig. 1).[23,29] A single treatment with LTβR-Ig temporarily dedifferentiates FDCs for $\approx$ 28 days.[29] FDCs trap and retain antigens on their surfaces through interactions between complement components and cellular complement receptors.[38,39] Furthermore, complement components C1q and C3, and cellular complement receptors are considered to play an important role in the localization of scrapie infectivity to FDCs.[40,41] In the present work, treatment with LTβR-Ig resulted in the temporary loss of expression of the FDC-associated molecules complement receptor 1 (CD35) and FDC-M2 in ILNs and the spleen. The epitope recognized by the FDC-M2-specific monoclonal antiserum has been identified as complement component C4.[42] Therefore, the temporary loss of FDC-M2-specific immunostaining after treatment with LTβR-Ig suggests that any remaining immature FDCs, if present, would be unable to trap and retain complement-opsonized antigens. These data suggest that these cells would also have lost their ability to capture scrapie during the period of dedifferentiation.

Within 17 days after scrapie inoculation by skin scarification, PrP^{Sc}-containing FDC networks were detected in the draining ILN of control mice, but not in the non-draining lymph node or spleen. By 45 days after inoculation, the number of FDC networks containing PrP^{Sc} had visibly increased in the draining ILN and were also detectable in a few networks in the non-draining ILN and spleen. The detection of low levels of PrP^{Sc} in the non-draining ILN and spleen at the later time-point suggests that following accumulation in the draining lymph node, PrP^{Sc} is disseminated to other lymphoid tissues via the bloodstream. In contrast, mice treated with LTβR-Ig at 42 days post-TSE inoculation had visibly reduced or undetectable accumulations of PrP^{Sc} within 3 days of treatment. The reduced detection of PrP^{Sc} coincided with the loss of FDCs following treatment with LTβR-Ig. The rapid reduction in PrP^{Sc} after treatment with LTβR-Ig is

probably a result of the release of PrP^{Sc} from dedifferentiating FDCs and its uptake and clearance by phagocytic cells, such as macrophages.[43–45]

Following scrapie inoculation via skin scarification, high levels of infectivity begin to peak in the draining ILN around 50 days postinoculation and subsequently plateau (Mohan *et al.* unpublished). In this study, ILNs and spleens from control mice contained high levels of scrapie infectivity at 70 days postinoculation. However, when mice were treated with LTβR-Ig 3 days before inoculation, scrapie infectivity was undetectable within the ILNs and spleen at 70 days postinoculation, ≈ 40 days after the expected reappearance of mature FDCs. Furthermore, in the temporary absence of FDCs at the time of inoculation, disease susceptibility was reduced and survival time in those treated mice that did develop clinical disease was significantly extended. Thus, these data suggest that scrapie infectivity from the inoculum is unable to replicate in the draining lymphoid tissue in the temporary absence of FDCs at the time of inoculation. The reduced disease susceptibility of LTβR-Ig-treated mice and absence of infectivity in lymphoid tissues at 70 days postinoculation suggests that the original inoculum is cleared by macrophages.[43–45] However, three LTβR-Ig-treated mice did develop clinical disease, albeit with incubation periods beyond the range observed in control, treated mice. Therefore, in some LTβR-Ig-treated mice, a fraction of the inoculum was able to persist until the FDCs reappeared.

The levels of infectivity responsible for natural TSE transmissions are unknown but are probably much lower than the moderate dose used in this study. SCID mice, which have a permanent absence of FDCs, do not develop clinical scrapie when inoculated via the skin with the same dose of scrapie used in the current study.[4,20] Therefore, the dose of scrapie infectivity administered in previous[4,20] and in the current work is not taken up directly by peripheral nerves, bypassing a need for replication in lymphoid tissues. The lack of detection of disease-specific PrP accumulations in the brains of surviving LTβR-Ig-treated mice demonstrates that, in our study, neuroinvasion following inoculation via the skin does not occur by direct uptake via nerves in the skin. These data also suggest that infection will have reached the CNS in the LTβR-Ig-treated mice that did develop clinical disease following replication on regenerated FDCs, and not by direct uptake by peripheral nerves. However, these data do not exclude the possibility, following inoculation with a higher dose of scrapie than the one used here, that neuroinvasion might occur via an FDC-independent pathway, such as uptake by peripheral nerves.

Treatment with LTβR-Ig 14 days after scrapie inoculation by skin scarification likewise significantly extended the survival time, but had no effect on disease susceptibility to the dose of scrapie used. PrP^{Sc} had already begun to accumulate upon FDC networks in the draining ILNs prior to treatment with LTβR-Ig at 14 days postinoculation. This suggests that the level of infectivity which had accumulated prior to treatment with LTβR-Ig was sufficient to avoid substantial clearance by phagocytic cells during the period of FDC dedifferentiation. The extended incubation period in these LTβR-Ig treated mice is probably related to the time required for the FDC networks to restore and initiate replication of the retained scrapie infectivity. This would delay the subsequent transfer of infectivity to peripheral nerves. Our data are also consistent with the hypothesis that the action of macrophages on TSE infectivity is concentration-dependent: the low concentrations derived from the original inoculum may be easily destroyed, whereas higher concentrations, such as those present in the ILN 14 days after inoculation, are less easily cleared and a fraction is retained.

Treatment with LTβR-Ig has no effect on disease pathogenesis once infection is established within the peripheral or central nervous systems.[30,46] The lack of any observable effect of LTβR-Ig treatment on disease pathogenesis, when given 42 days after scrapie inoculation, suggests that neuroinvasion had already occurred prior to treatment. Neuroinvasion may have occurred directly from the draining ILN, which had a heavy deposition of PrP^{Sc} at the time of treatment, whereas only limited amounts of PrP^{Sc} were detected in the spleen (Table 1). Removal of the spleen before i.p. scrapie inoculation significantly extends the survival time, suggesting that the spleen plays an important role in neuroinvasion via this route.[47] In contrast, removal of the spleen before subcutaneous scrapie inoculation has no effect on pathogenesis.[48] Data from these studies and the current study indicate that the major route of neuroinvasion following inoculation via the skin is not via the spleen.

Treatment with LTβR-Ig inhibits or prevents the development of experimental autoimmune encephalomyelitis by impairing T-lymphocyte responses and migration.[49] LIGHT is a transmembrane protein produced by activated T lymphocytes that also binds to LTβR.[50] However, the effects of treatment with LTβR-Ig on scrapie pathogenesis are unlikely to be a result of impaired LTβR- or LIGHT-mediated T-lymphocyte responses or migration, as pathogenesis is unaffected in T-lymphocyte-deficient mice.[51–54] Signalling via LTβR has been shown to be important for the presence of migratory dendritic cells in the spleen.[55] Therefore, it is plausible that blockade of the LTβR-signalling pathway might have affected cell trafficking or the transport of scrapie infectivity to the draining ILN. However, as treatment with LTβR-Ig 14 days after inoculation significantly extended the survival time, the effects of treatment on scrapie pathogenesis are unlikely to be the result of effects on cell trafficking, as dendritic cells migrate to draining lymphoid tissues within the first few hours of antigen encounter.[56] Migratory Langerhans' cells might also transport TSEs from the epidermis to the draining lymph node. However, as LTα- or LTβ-defici-

ency does not affect the distribution of Langerhans' cells in the skin, or their ability to take up antigen and migrate to the draining lymph node,[57] the effects of LTβR-Ig treatment on scrapie pathogenesis are also unlikely to be caused by impaired transportation by Langerhans' cells. Collectively, these observations suggest that it is highly unlikely that the major effects of LTβR-Ig treatment on scrapie pathogenesis are independent of its effects on FDC maturation.

Our data demonstrate that mature FDCs are critical for the accumulation of scrapie in the draining lymph node following inoculation by skin scarification. Furthermore, in the temporary absence of FDCs, disease susceptibility is reduced. Treatments that inactivate FDCs may have therapeutic potential against peripherally acquired TSEs. However, such treatments have no effect on pathogenesis once disease has spread from the FDCs to peripheral nerves.[58] While little is known about the precise timing of these events, comparisons of the effects of LTβR-Ig treatment on scrapie pathogenesis following inoculation by different peripheral routes indicate that this period varies considerably according to the route of exposure. Neuroinvasion will probably occur rapidly from the gastrointestinal tract because treatment with LTβR-Ig, 14 days after oral inoculation, is ineffective.[30] In the current study, our data suggest that neuroinvasion occurs between 14 and 42 days after inoculation via the skin. However, treatment with LTβR-Ig remains effective, even when administered up to 42 days after i.p. scrapie inoculation.[17,46] Thus, if manipulation of FDCs were to be used therapeutically against TSEs, the time interval available for intervention would depend critically on the route of TSE exposure.

Acknowledgments

This work was supported by funding from the Medical Research Council and the Biotechnology and Biological Sciences Research Council. We thank Irene McConnell, Mary Brady, Fraser Laing and Rebecca Greenan (Institute for Animal Health, Edinburgh, UK) for excellent technical support; Christine Farquhar (Institute for Animal Health) for provision of 1B3 polyclonal antiserum. LTβR-Ig and hu-Ig were kindly provided by DrTemp. Jeffrey Browning (Biogen Inc., Cambridge, MA) and requests for these reagents should be addressed to: Jeff_Browning@biogen.com

References

1 Duffy P, Wolf J, Collins G, DeVoe AG, Streffin B, Cowen D. Possible person to person transmission of Creutzfeldt–Jacob disease. *N Engl J Med* 1974; **290**:692.

2 Llewelyn CA, Hewitt PE, Knight RSG, Amar K, Cousens S, Mackenzie J, Will RG. Possible transmission of variant Creutzfeldt–Jakob disease by blood transfusion. *Lancet* 2004; **363**:417–21.

3 Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a *PRNP* codon 129 heterozygous patient. *Lancet* 2004; **354**:527–9.

4 Taylor DM, McConnell I, Fraser H. Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. *J Gen Virol* 1996; **77**:1595–9.

5 Bartz JC, Kincaid AE, Bessen RA. Rapid prion neuroinvasion following tongue infection. *J Virol* 2003; **77**:583–91.

6 Brotherston JG, Renwick CC, Stamp JT, Zlotnik I. Spread of scrapie by contact to goats and sheep. *J Comp Pathol* 1968; **78**:9–17.

7 Flechsig E, Hegyi I, Enari M, Scwarz P, Collinge J, Weissmann C. Transmission of scrapie by steel-surface-bound prions. *Mol Med* 2001; **7**:679–84.

8 Birmingham K. Were some CJD victims infected by vaccines? *Nature* 2000; **408**:3–5.

9 Lupi O. Prions in dermatology. *J Am Acad Dermatol* 2002; **46**:790–3.

10 Bolton DC, McKinley MP, Prusiner SB. Identification of a protein that purifies with the scrapie prion. *Science* 1982; **218**:1309–11.

11 Prusiner SB, Bolton DC, Groth DF, Bowman KA, Cochran SP, McKinley MP. Further purification and characterisation of scrapie prions. *Biochemistry (Mosc)* 1982; **21**:6942–50.

12 Brown KL, Stewart K, Ritchie D *et al.* Scrapie replication in lymphoid tissues depends on PrP-expressing follicular dendritic cells. *Nat Med* 1999; **5**:1308–12.

13 Mabbott NA, Williams A, Farquhar CF, Pasparakis M, Kollias G, Bruce ME. Tumor necrosis factor-alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie. *J Virol* 2000; **74**:3338–44.

14 van Keulen LJM, Schreuder BEC, Meloen RH, Mooij-Harkes G, Vromans MEW, Langeveld JPM. Immunohistological detection of prion protein in lymphoid tissues of sheep with natural scrapie. *J Clin Microbiol* 1996; **34**:1228–31.

15 Hill AF, Butterworth RJ, Joiner S *et al.* Investigation of variant Creutzfeldt–Jakob disease and other prion diseases with tonsil biopsy samples. *Lancet* 1999; **353**:183–9.

16 Sigurdson CJ, Williams ES, Miller MW, Spraker TR, O'Rourke KI, Hoover EA. Oral transmission and early lymphoid tropism of chronic wasting disease PrP^{res} in mule deer fawns (*Odocoileus hemionus*). *J Gen Virol* 1999; **80**:2757–64.

17 Montrasio F, Frigg R, Glatzel M, Klein MA, Mackay F, Aguzzi A, Weissmann C. Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. *Science* 2000; **288**:1257–9.

18 Glatzel M, Heppner FL, Albers KM, Aguzzi A. Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion. *Neuron* 2001; **31**:25–34.

19 Prinz M, Heikenwalder M, Junt T *et al.* Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion. *Nature* 2003; **425**:957–62.

20 Mohan J, Brown KL, Farquhar CF, Bruce ME, Mabbott NA. Scrapie transmission following exposure through the skin is dependent on follicular dendritic cells in lymphoid tissues. *J Dermatol Sci* 2004; **35**:101–11.

21 Kapasi ZF, Burton GF, Schultz LD, Tew JG, Szakal AK. Induction of functional follicular dendritic cell development in severe combined immunodeficiency mice. *J Immunol* 1993; **150**:2648–58.

22 Aucouturier P, Geissmann F, Damotte D *et al.* Infected splenic dendritic cells are sufficient for prion transmission to the CNS in mouse scrapie. *J Clin Invest* 2001; **108**:703–8.

23 Force WR, Walter BN, Hession C, Tizard R, Kozak CA, Browning JL, Ware CF. Mouse lymphotoxin-beta receptor. *J Immunol* 1995; **155**:5280–8.

24 Fraser H, Dickinson AG. Agent-strain differences in the distribution and intensity of grey matter vacuolation. *J Comp Pathol* 1973; **83**:29–40.

25 Dickinson AG, Meikle VM, Fraser H. Genetical control of the concentration of ME7 scrapie agent in the brain of mice. *J Comp Pathol* 1969; **79**:15–22.

26 McBride P, Eikelenboom P, Kraal G, Fraser H, Bruce ME. PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. *J Pathol* 1992; **168**:413–8.

27 Schulz-Schaeffer WJ, Tschoke S, Kranefuss N *et al.* The paraffin-embedded tissue blot detects PrP^{sc} early in the incubation time in prion diseases. *Am J Pathol* 2000; **156**:51–6.

28 Farquhar CF, Somerville RA, Ritchie LA. Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *J Virol Methods* 1989; **24**:215–22.

29 Mackay F, Browning JL. Turning off follicular dendritic cells. *Nature* 1998; **395**:26–7.

30 Mabbott NA, Young J, McConnell I, Bruce ME. Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility. *J Virol* 2003; **77**:6845–54.

31 Farquhar CF, Dornan J, Somerville RA, Tunstall AM, Hope J. Effect of *Sinc* genotype, agent isolate and route of infection on the accumulation of protease-resistant PrP in non-central nervous system tissues during the development of murine scrapie. *J Gen Virol* 1994; **75**:495–504.

32 Kosco-Vilbois MH, Zentgraf H, Gerdes J, Bonnefoy J-Y. To 'B' or not to 'B' a germinal center? *Immunol Today* 1997; **18**:225–30.

33 Chaplin DD, Fu Y-X. Cytokine regulation of secondary lymphoid organ development. *Curr Opin Immunol* 1998; **10**:289–97.

34 Matsumoto M, Lo SF, Carruthers CJL *et al.* Affinity maturation without germinal centres in lymphotoxin-α-deficient mice. *Nature* 1996; **382**:462–6.

35 Koni PA, Sacca R, Lawton P, Browning JL, Ruddle NH, Flavell RA. Distinct roles in lymphoid organogenesis for lymphotoxins α and β revealed in lymphotoxin β-deficient mice. *Immunity* 1997; **6**:491–500.

36 Endres R, Alimzhanov MB, Plitz T *et al.* Mature follicular dendritic cell networks depend on expression of lymphotoxin β receptor by radioresistant stromal cells and of lymphotoxin β and tumour necrosis factor by B cells. *J Exp Med* 1999; **189**:159–68.

37 Fütterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* 1998; **9**:59–70.

38 Yoshida K, van den Berg TK, Dijkstra CD. Two functionally different follicular dendritic cells in secondary lymphoid follicles of mouse spleen, as revealed by CR1/2 and FcRγII-mediated immune-complex trapping. *Immunology* 1993; **80**:34–9.

39 Nielsen CH, Fischer EM, Leslie RGQ. The role of complement in the acquired immune response. *Immunology* 2000; **100**:4–12.

40 Klein MA, Kaeser PS, Schwarz P *et al.* Complement facilitates early prion pathogenesis. *Nat Med* 2001; **7**:488–92.

41 Mabbott NA, Bruce ME, Botto M, Walport MJ, Pepys MB. Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie. *Nat Med* 2001; **7**:485–7.

42 Taylor PR, Pickering MC, Kosco-Vilbois MH, Walport MJ, Botto M, Gordon S, Martinez-Pomares L. The follicular dendritic cell restricted epitope, FDC-M2, is complement C4; localization of immune complexes in mouse tissues. *Eur J Immunol* 2002; **32**:1883–96.

43 Carp RI, Callahan SM. *In vitro* interaction of scrapie agent and mouse peritoneal macrophages. *Intervirology* 1981; **16**:8–13.

44 Carp RI, Callahan SM. Effect of mouse peritoneal macrophages on scrapie infectivity during extended *in vitro* incubation. *Intervirology* 1982; **17**:201–7.

45 Beringue V, Demoy M, Lasmezas CI *et al.* Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis. *J Pathol* 2000; **190**:495–502.

46 Mabbott NA, Mackay F, Minns F, Bruce ME. Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. *Nat Med* 2000; **6**:719–20.

47 Fraser H, Dickinson AG. Pathogenesis of scrapie in the mouse: the role of the spleen. *Nature* 1970; **226**:462–3.

48 Fraser H, Bruce ME, Davies D, Farquhar CF, McBride PA. The lymphoreticular system in the pathogenesis of scrapie. In: Prusiner, SB, Collinge, J, Powell, J, Anderton, B, eds. *Prion Diseases of Humans and Animals*. Chichester, UK: Ellis Harwood Ltd, 1992:309–17.

49 Gommerman JL, Giza K, Perper S, Sizing I, Ngam-ek A, Nickerson-Nutter C, Browning JL. A role for surface lymphotoxin in experimental autoimmune encephalomyelitis independent of LIGHT. *J Clin Invest* 2003; **112**:755–67.

50 Mauri DN, Ebner R, Montgomery RI *et al.* LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for Herpesvirus entry mediator. *Immunity* 1998; **8**:21–30.

51 McFarlin DE, Raff MC, Simpson E, Nehlsen SH. Scrapie in immunologically deficient mice. *Nature* 1971; **233**:336.

52 Fraser H, Dickinson AG. Studies on the lymphoreticular system in the pathogenesis of scrapie: the role of spleen and thymus. *J Comp Pathol* 1978; **88**:563–73.

53 Klein MA, Frigg R, Flechsig E *et al.* A crucial role for B cells in neuroinvasive scrapie. *Nature* 1997; **390**:687–91.

54 Klein MA, Frigg R, Raeber AJ *et al.* PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nat Med* 1998; **4**:1429–33.

55 Wu Q, Wang Y, Hedgeman EO, Browning JL, Fu Y-X. The requirement of membrane lymphotoxin for the presence of dendritic cells in lymphoid tissues. *J Exp Med* 1999; **190**:629–38.

56 Banchereau J, Briere F, Caux C *et al.* Immunobiology of dendritic cells. *Annu Rev Immunol* 2000; **18**:767–811.

57 Rennert PD, Hochman PS, Flavell RA, Chaplin DD, Jayaraman S, Browning JL, Fu Y-X. Essential role of lymph nodes in contact hypersensitivity revealed in lymphotoxin-α-deficient mice. *J Exp Med* 2001; **193**:1227–38.

58 Mabbott NA, Bruce ME. Follicular dendritic cells as targets for intervention in transmissible spongiform encephalopathies. *Semin Immunol* 2002; **14**:285–93.